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Preface to the Fourth Edition

This edition has been overdue and in spite of our best efforts we could not get it out earlier. We have made considerable revisions in many chapters, specially in the fields of immunology and virology. We have devoted a separate chapter to retroviruses, HIV taking a prominent place. We owe much to those who helped us with their criticisms and suggestions and we desire our readers to continue to render us such services.

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Preface to the Third Edition

Rapid developments in microbiology in recent years necessitated this new edition. Brief notes on important advances had been included in the reprints which had to be brought out every year, but that was not entirely satisfactory. In this edition we have updated all chapters and revised most. A section on Bacteriology of Air and a chapter on Hospital Infections have been added. Recent publications have been indicated in the suggestions for further reading. Every effort has been made to see that significant advances in the various fields of microbiology have not been missed.

We thank again the students and teachers of microbiology for their helpful comments and criticisms. We welcome suggestions for improving the book.

R. Ananthanarayan
C.K.Jayaram Paniker

Preface to the Second Edition

We owe a deep debt of gratitude to the students of and teachers in microbiology in India for the splendid reception they have given to our book. The first edition was sold out in four months and a reprint could be brought out only in 1979. Before we could complete our revision, a revised reprint incorporating minimal essential newer knowledge had to be brought out in 1980.

The second edition has been thoroughly revised. The chapters on Immunodeficiency diseases, Rhabdoviruses, Hepatitis viruses and Bacteriology of Water and Milk have been completely rewritten. Chapters on Acute Diarrhoeal Diseases, Immunoprophylaxis and Immunotherapy have been added. Mycoplasma and Actinomycetes have been dealt with in separate chapters. Much newer knowledge has been added. A number of diagrams have been replaced by better ones and additional diagrams including electromicroscopic pictures have been introduced. Each chapter has at its end a list for further reading.

We request our readers to send us their comments and suggestions for further improving the book.

R. Ananthanarayan
C.K. Jayaram Paniker

Preface to the First Edition

Many of the health problems in developing countries like India are different from those of developed countries. Bacterial diseases still play a considerable role in diseases in our country. Topics such as cholera and enteric diseases are important to us though only of less or academic interest to the developed countries. The increasing importance of the newer knowledge in immunology to health and disease is not adequately stressed in most of the extant textbooks. Virus diseases which are responsible for nealy 60 per cent of human illness require wider coverage. The general approach to the teaching of Microbiology in our country has also been rather static. All these factors called for a textbook of Microbiology more suited to countries like India.

We therefore undertook this endeavour based on our experience of teaching undergraduates and postgraduates for over two decades. We omitted the discipline of parasitology from our book since we already have an excellent textbook on the subject published in India.

This book has taken us over three years to write and over a year in publication. Naturally we would be out of date to a certain and inevitable extent. We do not claim any perfection. On the contrary, we have requested medical students and teachers all over the country to write to us about any shortcomings and give us suggestions as to how to improve the book. We shall spare no pains in seeing that their valuable suggestions are given effect to in our second edition.

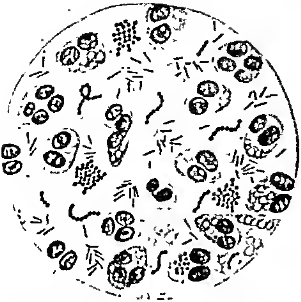
R. Ananthanarayan
C.K. Jayaram Paniker

Staph; Strept; Hypers; Infection, Immune Response; Ab & funcn.

Acknowledgements

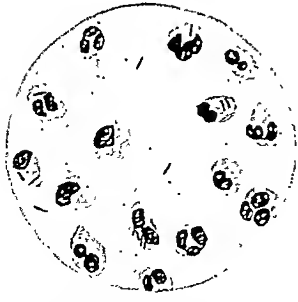
We express our indebtedness to Professor M. Mathen, Christian Medical College, Vellore, S. India, for Figs. 53.2 and 60.1; Dr. Paul A. Blake, Deputy Chief, Enteric Diseases Branch, CDC, Atlanta Georgia, for Fig. 33.2; Dr. J. Shanmugham, Sri Chitra Tirunal Medical Centre, Trivandrum, for Fig. 48.4; National Institute of Virology, Pune, for Fig. 57.1 and Dr. M. Satyavati M. Sirsat, Ultrastructure Division, Cancer Research Institute, Parel, Bombay, for Fig. 61.6.

PLATE I



Gram stain of a pus smear. Plate shows Gram positive violet coloured cocci in groups (staphylococci) in chains (streptococci) and pink rods (Gram negative bacilli). Pus cells show up pink stained.

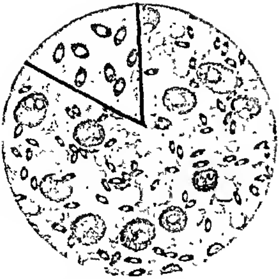
Gonococci in urethral discharge. Gram stain.



Acid fast stain (Ziehl Neelsen stain) of sputum. The red rods are *M. tuberculosis*

M. leprae. Ziehl Neelsen stain of section of lepra nodule showing lepra cells and characteristic arrangement of

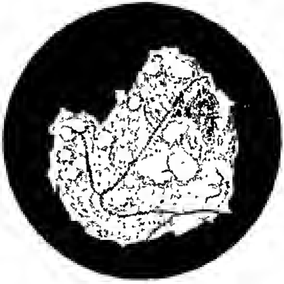
PLATE II



Yersinia pestes Smear from enlarged lymph gland from a case of plague Leishman stain Characteristic bipolar staining

Negri bodies in dog's brain in rabies (hippocampus of dog) Alcoholic eosin and methylene blue stain

Allantoic membrane showing Variola pocks Clear cut and white



Allantoic membrane showing Vaccinia pocks Note irregularity in shape and size

Part I

1 Historical Introduction

Medical microbiology deals with the causative agents of infectious diseases of man, his reactions to them and the methods of protection against such diseases.

Disease and death have always attracted the attention of the human mind. Ancient man ascribed them to divine wrath and other supernatural forces. Later, other concepts such as the influence of environment, of bodily constitution and of faulty diet were proposed. There have been, from very early times, occasional suggestions that diseases may result from invasion of the body by external contagion. Varo and Columella in the first century B.C. postulated that diseases were caused by invisible beings *(Animalia minuta)*, inhaled or ingested. Fracastorius of Verona (1546) proposed a *contagium vivum* as the possible cause of infectious disease and von Plenciz (1762) suggested that each disease was caused by a separate agent. Kircher (1659) reported finding minute worms in the blood of plague victims, but with the equipment available to him it is more likely that what he observed

Leeuwenhoek the world of 'little animalcules' represented only a curiosity of nature. It was only some two centuries later that their importance in medicine and in other areas of biology came to be recognised.

The earliest discovery of a pathogenic microorganism was probably made by Augustino Bassi (1835), who showed that the muscardine disease of silk worms was caused by a fungus. Davaine and Pollender (1850) observed anthrax bacilli in the blood of animals dying of the disease. In fact, even before the microbial aetiology of infections had been established, this was evident to some observant physicians. Oliver Wendell Holmes in the U.S.A. (1843) and Ignaz Semmelweis in Vienna (1846) had independently concluded that puerperal sepsis was transmitted by the contaminated hands of obstetricians and medical students and demonstrated the efficacy of simple measures such as washing hands in an antiseptic solution.

The development of bacteriology as a scientific discipline dates from Louis Pasteur (1822–95). Though trained as a chemist, his studies on fermentation led him to take an interest in microorganisms. He established that fermentation was the result of microbial activity and that different types of fermentations were associated with the activity of different kinds of microorganisms (1857). The basic principles and techniques of bacteriology were evolved by Pasteur during his enquiry into the origin of microbes. This was then the subject of much controversy. Needham, an Irish priest, had in 1745 published experiments purporting the spontaneous generation (abio-

genesis) of microorganisms in putrescible fluids. This view was opposed by Spallanzani, an Italian abbot (1769). In a series of classic experiments, Pasteur proved conclusively that all forms of life, even microbes, arose only from their like and not *de novo*. In the course of these studies, he introduced techniques of sterilisation and developed the steam steriliser, hot-air oven and autoclave. He also established the differing growth needs of different bacteria. His work attracted such attention and he attained such eminence in the world of science that not only France but all Europe looked to him to solve major problems in various fields. Thus started his studies on pebrine, anthrax, chicken cholera, and hydrophobia. An accidental observation that chicken cholera bacillus cultures left on the bench for several weeks lost their pathogenic property but retained their ability to protect the birds against subsequent infection by them led to the discovery of the process of attenuation and the development of live vaccines. He attenuated cultures of the anthrax bacillus by incubation at high temperature (42°–43°C) and proved that inoculation of such cultures in animals induced specific protection against anthrax. The success of such immunisation was dramatically demonstrated by a public experiment on a farm at Pouilly-le-Fort (1881) during which vaccinated sheep, goats and cows were challenged with a virulent anthrax bacillus culture. All the vaccinated animals survived the challenge while an equal number of unvaccinated control animals succumbed to it. It was Pasteur who coined the term *vaccine* for such prophylactic preparations to commemorate the first of such preparations, namely, cowpox, employed by Jenner for protection against smallpox.

The greatest impact in medicine was made by Pasteur's development of a vaccine for hydrophobia. This was acclaimed throughout the world. The Pasteur Institute, Paris, was built by public contribution and similar institutions were established soon in many other countries for the preparation of vaccines and for the investigation of infectious diseases.

An immediate application of Pasteur's work was the introduction of antiseptic techniques in surgery by Lister (1867) effecting a pronounced drop in mortality and morbidity due to surgical sepsis. Lister's antiseptic surgery involving the use of carbolic acid was cumbersome and hazardous, but was a milestone in the evolution of surgical practice from the era of 'laudable pus' to modern aseptic techniques.

While Pasteur in France laid the foundations of microbiology, Robert Koch (1843–1910) in Germany perfected bacteriological techniques during his studies on the culture and characters of the anthrax bacillus (1876). He introduced staining techniques and methods of obtaining bacteria in pure culture using solid media. He discovered the bacillus of tuberculosis (1882) and the cholera vibrio (1883).

Pasteur and Koch attracted many gifted disciples who discovered the causative agents of several bacterial infections and enlarged the scope and content of microbiology by their labours. In 1874, Hansen described the leprosy bacillus; in 1879, Neisser described the gonococcus; in 1881, Ogston discovered the staphylococcus; in 1884, Loeffler isolated the diphtheria bacillus; in 1884, Nicolaier observed the tetanus bacillus in soil; in 1886, Fraenkel described the pneumococcus; in 1887, Bruce identified the causative agent of Malta fever; in 1905, Schaudinn and Hoffmann discovered the spirochaete of syphilis.

Roux and Yersin (1888) identified a new mechanism of pathogenesis when they discovered the diphtheria toxin. Similar toxins were identified in tetanus and some other bacteria. The toxins were found to be specifically neutralised by their antitoxins. Ehrlich who studied toxins and antitoxins in quantitative terms laid the foundations of biological standardisation.

The causative agents of various infectious diseases were being reported by different investigators in such profusion that it was necessary to introduce criteria for proving the claims that a microorganism isolated from a disease was indeed causally related to it. These criteria, first indicated by Henle, were enunciated by Koch and are known as Koch's postulates. According

to these, a microorganism can be accepted as the causative agent of an infectious disease only if the following conditions are satisfied:

1. The bacterium should be constantly associated with the lesions of the disease.
2. It should be possible to isolate the bacterium in pure culture from the lesions.
3. Inoculation of such pure culture into suitable laboratory animals should reproduce the lesions of the disease.
4. It should be possible to reisolate the bacterium in pure culture from the lesions produced in the experimental animals.

An additional criterion introduced subsequently requires that specific antibodies to the bacterium should be demonstrable in the serum of patients suffering from the disease. Though it may not always be possible to satisfy all the postulates in every case, they have proved extremely useful in sifting doubtful claims made regarding the causative agents of infectious diseases.

By the beginning of the twentieth century, many infectious diseases had been proved to have been caused by bacteria. But there remained a large number of diseases such as smallpox, chickenpox, measles, influenza and the common cold for which no bacterial cause could be established. During his investigation of rabies in dogs, Pasteur had suspected that the disease could be caused by a microbe too small to be seen even under the microscope. The existence of such ultramicroscopic microbes was proved when Ivanovsky (1892) reproduced mosaic disease in the tobacco plant, by applying to healthy leaves juice from the diseased plants from which all bacteria had been removed by passage through fine filters. Beijerinck (1898) confirmed these findings. Loeffler and Frosch (1898) observed that the foot and mouth disease of cattle was caused by a similar filter-passing virus. The first human disease proved to have a virus aetiology was yellow fever. The U.S. Army Commission under Walter Reed, investigating yellow fever in Cuba (1902), established not only that it was caused by a filterable virus but also that it was transmitted through the bite of infected mosquitoes. Landsteiner and Popper (1909) showed that poliomyelitis was caused by a filterable virus and transmitted the disease experimentally to monkeys. Investigation of viruses and the diseases caused by them was rendered difficult as viruses could not be visualised under light microscopes or grown in culture media. Though the larger viruses could be seen after appropriate staining under the light microscope, detailed study of their morphology had to wait till the introduction of the electron microscope by Ruska (1934) and subsequent refinements in electron microscopic techniques. Cultivation of viruses was possible only in animals or in human volunteers till the technique of growing them on chick embryos was developed by Goodpasture in the 1930s. The application of tissue culture in virology expanded the scope of virological techniques considerably.

The possibility that virus infection could lead to malignancy was first put forth by Ellerman and Bang (1908). Peyton Rous (1911) isolated a virus causing sarcoma in fowls. Several viruses have since been isolated which cause natural and experimental tumours in animals and birds. Viruses also cause malignant transformation of infected cells in tissue culture. The discovery of viral and cellular oncogenes has shed light on the possible mechanisms of viral oncogenesis. After many decades of futile search, positive proof of the viral causation of human malignancy was established when the virus of human T-cell leukaemia was isolated in 1980.

Twort (1915) and d'Herelle (1917) independently discovered a lytic phenomenon in bacterial cultures. The agents responsible were termed bacteriophages — viruses that attack bacteria. Early hopes that bacteriophages may have therapeutic applications had to be abandoned, but these viruses have paid unexpected scientific dividends. The essential part of viruses is their core of nucleic acid which acts as the carrier of genetic information in the same manner as in higher organisms. The discipline of molecular biology owes its origin largely to studies on the genetics of bacteriophages and bacteria.

It had been noticed from very early days that

persons surviving an attack of smallpox did not develop the disease when exposed to the infection subsequently. This observation had been applied for the prevention of the disease by producing a mild form of smallpox intentionally (variolation). This practice, prevalent in India, China and other ancient civilisations from time immemorial, was introduced into England by Lady Mary Wortley Montague (1718) who had observed the custom in Turkey. Variolation was effective but hazardous. Jenner observing the immunity to smallpox in milkmaids who were liable to occupational cowpox infection introduced the technique of vaccination using cowpox material (1796). This was the first instance of scientific immunisation and, though introduced empirically, has stood the test of time. Jenner's vaccination paved the way for the ultimate eradication of smallpox.

The next major discovery in immunity was Pasteur's development of vaccines for chicken cholera, anthrax and rabies. While the techniques introduced by him were successful, the mechanism of protection afforded by them remained obscure. The explanation of the underlying mechanism came from two sources. Nuttall (1888) observed that defibrinated blood had a bactericidal effect, and Buchner (1889) noticed that this effect was abolished by heating the sera for one hour at 55°C. The heat labile bactericidal factor was termed 'alexine'. A specific humoral factor or 'antibody' was described by von Behring and Kitasato (1890) in the serum of animals which had received sublethal doses of tetanus toxin. Pfeiffer (1893) demonstrated bactericidal effect *in vivo* by injecting live cholera vibrios intraperitoneally in guinea pigs previously injected with killed vibrios. The vibrios were shown to undergo lysis. The humoral nature of such lytic activity was proved by Bordet (1895), who defined the two components participating in the reaction, the first being heat stable and found in immune sera (antibody or *substance sensibilicatrice*) and the second being heat labile and identical with Buchner's alexine, subsequently named 'complement'. Soon a number of other ways were demonstrated in which antibodies react with antigens, such as agglutination, precipitation, complement fixation and neutralisation.

Metchnikoff (1883) discovered the phenomenon of phagocytosis and proposed the phagocytic response as the prime defence against the microbial invasion of tissues. This led to the cellular concept of immunity. Polemics regarding the significance of the cellular and humoral mechanisms of immunity were largely put to rest with the discovery by Wright (1903) of opsonisation, in which antibodies and phagocytic cells act in conjunction.

Prior experience with a microorganism or other antigen did not always result in the beneficial effect of immunity or protection. At times it caused the opposite effect. Koch (1890) had noticed that when the tubercle bacillus or its protein was injected into a guinea pig already infected with the bacillus, an exaggerated response took place — a hypersensitivity reaction known as Koch's phenomenon. Portier and Richet (1902), studying the effect of the toxic extracts of sea anemones in dogs made 'the paradoxical observation that dogs which had prior contact with the toxin were abnormally sensitive to even minute quantities of it subsequently.' This phenomenon was termed 'anaphylaxis'. Later, many similar reactions were observed, both experimentally and in nature, of injury, disease or even death resulting from repeated contacts with antigens. The importance of this phenomenon, in the pathogenesis of many human diseases, led to the development of the discipline of 'allergy'.

The characteristic feature of immunity, whether it is protective, or destructive as in allergy, is its specificity. As the mediators of humoral immunity (antibodies) are globulins, the explanation for the exquisite specificity of the immunological reaction had to await the advances in protein chemistry. The pioneering work of Landsteiner laid the foundations of immunochemistry. Chemists dominated the study of immunity for several decades, and theories of antibody synthesis were postulated by

them, which sometimes ran counter to biological laws. In 1955, Jerne proposed the 'natural selection' theory of antibody synthesis which attempted to explain the chemical specificity and biological basis of antibody synthesis, signifying a return to the original views of antibody formation proposed by Ehrlich (1898). Burnet (1957) modified this into the clonal selection theory, a concept which, with minor alterations, holds sway even now. The last two decades have witnessed an explosion of conceptual and technical advances in immunology. Immunological processes in health and disease are now better understood following the identification of the two components of immunity — the humoral or antibody mediated processes and the cellular or cell mediated processes — which develop and are manifested in separate pathways.

Till recently, a teleological view of immunity prevailed. It was considered a protective mechanism designed to defend the body against invasion by microorganisms. Based on the original suggestion of Thomas (1959), Burnet (1967) developed the concept of 'immunological surveillance', according to which the primary function of the immune system is to preserve the integrity of the body, seeking and destroying all 'foreign' antigens, whether autogenous or external in origin. Malignancy was visualised as a failure of this function, and the scope of immunity was enlarged to include natural defence against cancer. Another aspect of this role of immunity is in the rejection of homografts. Understanding of the immunological basis of transplantation, due largely to the work of Medawar and Burnet, made successful transplants possible by elective immunosuppression and proper selection of donors based on histocompatibility. The history of transplantation thus runs parallel to the history of blood transfusion, which was unsuccessful and even fatal before the discovery of blood groups by Landsteiner (1900).

During the early part of the 20th century, attempts were made to exploit the immunological information available by the development of vaccines and sera for the prophylaxis and treatment of infectious diseases. Till Domagk (1935) initiated scientific chemotherapy with the discovery of prontosil, antisera were the only specific therapeutic agents available for the management of infectious diseases.

Fleming (1929) made the accidental discovery that the fungus *Penicillium* produces a substance which destroys staphylococci. It was only the dire needs brought about by the Second World War that led to the isolation of the active substance penicillin and its subsequent mass production. This was the beginning of the antibiotic era, and other similar antibiotics were discovered in rapid succession. With the sudden availability of a wide range of antibiotics with potent antibacterial activity, it was hoped that bacterial infections would be controlled within a short period. But, soon, the development of drug resistance in bacteria presented serious difficulties.

With the development of a wide variety of antibiotics active against the whole spectrum of pathogenic bacteria, and of effective vaccines against most viral diseases, expectations were raised about the eventual elimination of all infectious diseases. The global eradication of smallpox inspired visions of similar campaigns against other major pestilences. However, such hopes were dampened when new infectious diseases began to appear, caused by hitherto unknown microorganisms or by known microbes, producing novel manifestations. The climax came in 1981 when AIDS was identified in the USA and began its pandemic spread. Unceasing vigilance appears essential to protect man from microbes.

Apart from the obvious benefits such as specific methods of diagnosis, prevention and control of infectious diseases, medical microbiology has contributed to scientific knowledge and human welfare in many other ways. Microorganisms constitute the smallest forms of living beings and, therefore, have been employed as models of studies on genetics and biochemistry. As nature's laws are universal in application, information derived from the investigation of microbes holds true, in the main, for man as well.

Studies on microorganisms have contributed,

more than anything else, to unravelling the genetic code and other mysteries of biology at the molecular level. They have made available information and techniques that could be used for genetic manipulation and molecular engineering.

The number of Nobel Laureates in Medicine and Physiology, awarded the prize for their work in microbiology, listed below, is evidence of the positive contribution made to human health by the science of microbiology.

1901 Emil A. Von Behring
1902 Sir Ronald Ross
1905 Robert Koch
1907 C L.A. Laveran
1908 Paul Ehrlich and Elie Metchnikoff
1913 Charles Richet
1919 Jules Bordet
1920 Johannes Fibiger
1928 Charles Nicolle
1930 Karl Landsteiner
1939 Gerhardt Domagk
1945 Sir Alexander Fleming, Sir Howard Florey and E B. Chain
1951 Max Theiler
1952 Selman A. Waksman
1954 J.F. Enders, F.C. Robbins and T.H. Weller
1958 G.W. Beadle, Joshua Lederberg and E.L. Tatum
1960 Sir Macfarlane Burnet and Sir Peter Brian Medawar
1965 Francois Jacob, Andre Lwoff and Jacques Monod
1966 Peyton Rous
1969 Max Delbruck, A.D. Hershey and Salvador Luria
1972 Gerald Edelman and Rodney Porter
1975 David Baltimore, Renato Dulbecco and Howard M. Temin
1976 Baruch S. Blumberg and Carleton Gajdusek
1980 Baruj Benacerraf, Jean Dausset and George Snell
1984 Niels Jerne, Cesar Milstein and Georges Kohler
1987 Susumu Tonegawa
1989 J. Michael Bishop and Harold E. Varmus

Further Reading

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2 Morphology and Physiology of Bacteria

Microorganisms are a heterogeneous group of several distinct classes of living beings. They were originally classified under the plant and animal kingdoms. As this proved unsatisfactory, a third kingdom, the *protista*, was formed for them. Based on differences in cellular organisation and biochemistry, the kingdom *protista* has been divided into two groups — *prokaryotes* and *eukaryotes*. Bacteria and blue-green algae are prokaryotes, while fungi, algae, slime moulds and protozoa are eukaryotes.

Bacteria are prokaryotic microorganisms that

TABLE 2.1

Some differences between prokaryotic and eukaryotic cells

Character	Prokaryotes (bact, B-G algae)	Eukaryotes (fungi, algae, slime moulds, proto...)
Nucleus:		
Nuclear membrane	Absent	Present
Nucleolus	Absent	Present
Deoxyribonucleoprotein	Absent	Present
Chromosome	One haploid (circular) double stranded DNA	More than one (linear)
Mitotic division	Absent	Present
Cytoplasm:		
Cytoplasmic streaming	Absent	Present
Pinocytosis	Absent	Present
Mitochondria	Absent	Present
Lysosomes	Absent	Present
Golgi apparatus	Absent	Present
Endoplasmic reticulum	Absent	Present
Chemical composition:		
Sterols	Absent	Present
Muramic acid	Present	Absent
Diaminopimelic acid	Present in some	Absent

do not contain chlorophyll. They are unicellular and do not show true branching, except in the so-called 'higher bacteria' (*Actinomycetales*).

Size of bacteria

The unit of measurement used in bacteriology is the micron (micrometre).
1 micron (μ) or micrometer (μm) = one thousandth of a millimetre.
1 millimicron (mμ) or nanometre (nm) = one thousandth of a micron or one millionth of a millimetre.
1 Angstrom unit (Å) = one tenth of a nanometre.

The limit of resolution with the unaided eye is about 200 microns. Bacteria, being much smaller, can be visualised only under magnification. Bacteria of medical importance generally measure 0.2–1.5 μ in diameter and about 3–5 μ in length.

Microscopy

The morphological study of bacteria requires the use of microscopes. Microscopy has come a long way since Leeuwenhoek first observed bacteria three hundred years ago using hand ground lenses. The following types of microscopes are being employed now.

Optical or light microscope Bacteria may be examined under the compound microscope, either in the living state or after fixation and staining. Examination of wet films or 'hanging drops' indicates the shape, arrangement, motility and approximate size of the cells. But due to lack of contrast, details cannot be appreciated

Phase contrast microscopy improves the contrast and makes evident the structures within cells that differ in thickness or refractive index. Also, the differences in refractive index between bacterial cells and the surrounding medium make them clearly visible. Retardation, by a fraction of a wavelength, of the rays of light that pass through the object, compared to the rays passing through the surrounding medium, produces 'phase' differences between the two types of rays. In the phase contrast microscope, 'phase' differences are converted into differences in intensity of light, producing light and dark contrast in the image.

Dark field (dark ground) microscope: Another method of improving the contrast is the dark field (dark ground) microscope in which reflected light is used instead of the transmitted light used in the ordinary microscope The essential part of the dark field microscope is the dark field condenser with a central circular stop, which illuminates the object with a cone of light, without letting any ray of light fall directly on the objective lens. Light rays falling on the object are reflected or scattered on to the objective lens, with the result that the object appears self-luminous against a dark background. The contrast gives an illusion of increased resolution, so that very slender organisms such as spirochaetes, not visible under ordinary illumination, can be clearly seen under the dark field microscope.

The resolving power of the light microscope is limited by the wavelength of light. In order to be seen and delineated (resolved), an object has to have a size of approximately half the wavelength of the light used. With visible light, using the best optical systems, the limit of resolution is about 300 nm. If light of shorter wavelength is employed, as in the ultraviolet microscope, the resolving power can be proportionately extended

Two specialised types of microscopes are: 1) *the interference microscope* which not only reveals cell organelles, but also enables quantitative measurements of the chemical constituents of cells such as lipids, proteins and nucleic acids, and 2) *the polarisation microscope* which enables the study of intracellular structures using differences in birefringence.

Electron microscope: In the electron microscope a beam of electrons is employed instead of the beam of light used in the optical microscope. The electron beam is focused by circular electromag-

nets, which are analogous to the lenses in the light microscope. The object which is held in the path of the beam scatters the electrons and produces an image which is focused on a fluorescent viewing screen. As the wavelength of electrons used is approximately 0.005 nm, as compared to 500 nm with visible light, the resolving power of the electron microscopes should be theoretically 100,000 times that of light microscopes. But in practice, the resolving power is about 0.1 nm.

The technique of shadow-casting with vaporised heavy metals has made possible pictures with good contrast and three-dimensional effect. Another valuable technique in studying fine structure is negative staining with phosphotungstic acid.

Gas molecules scatter electrons, and it is, therefore, necessary to examine the object in a vacuum. Hence, only dead and dried objects can be examined in the electron microscope. This may lead to considerable distortion in cell morphology. A method introduced to overcome this disadvantage is freeze-etching, involving the deep-freezing of specimens in a liquid gas and the subsequent formation of carbon-platinum replicas of the material. Since such frozen cells may remain viable, it is claimed that freeze-etching enables the study of cellular ultrastructure as it appears in the living state. The recent development of very high voltage electron microscopes may render possible the eventual examination of live objects. The scanning electron microscope is a recent innovation which permits the study of cell surfaces with greater contrast and higher resolution than with the shadow-casting technique.

Stained preparations

Live bacteria do not show much structural detail under the light microscope due to lack of contrast. Hence it is customary to use staining techniques to produce colour contrast. Bacteria may be stained in the living state. However, many dyes are toxic and kill the cell on staining. This type of staining during which the cell is killed is known as supravital staining. Nontoxic staining, during which cells retain their viability, is known as vital (or intravital) staining. Routine methods of staining bacteria employ drying and fixation of smears — procedures that kill them. Bacteria have an affinity for basic dyes due to the acidic nature of their protoplasm. Staining techniques in common use in bacteriology are the following:

1. Simple stains: Dyes such as methylene blue or basic fuchsin are used for simple staining. They provide colour contrast, but impart the same colour to all bacteria

2. Negative staining: Here, bacteria are mixed with dyes such as Indian ink or nigrosin that provide a uniformly coloured background against which the unstained bacteria stand out in contrast. This is particularly useful in the demonstration of bacterial capsules which do not take simple stains. Very slender bacteria such as spirochaetes that are not demonstrable by simple staining methods can be visualised by negative staining.

3. Impregnation methods: Cells and structures too thin to be seen under the ordinary microscope may be rendered visible if they are thickened by impregnation of silver on the surface. Such methods are used for the demonstration of spirochaetes and bacterial flagella.

4. Differential stains: These stains impart different colours to different bacteria or bacterial structures. The two most widely used differential stains are the Gram stain and the acid fast stain.

The Gram stain was originally devised by the histologist Christian Gram (1884) as a method of staining bacteria in tissues. The staining technique consists essentially of four steps:

1. primary staining with a pararosaniline dye such as crystal violet, methyl violet or gentian violet;
2. application of a dilute solution of iodine;

3. decolourisation with an organic solvent such as ethanol, acetone or aniline;
4. counterstaining with a dye of contrasting colour, such as carbol fuchsin, safranine or neutral red.

The Gram stain differentiates bacteria into two broad groups. Gram positive bacteria are those that resist decolourisation and retain the primary stain, appearing violet. Gram negative bacteria are decolourised by organic solvents and, therefore, take the counterstain, appearing red. The exact mechanism of the Gram reaction is not understood. The Gram positive cells have a more acidic protoplasm, which may account for their retaining the basic primary dye more strongly than the Gram negative bacteria. Decolourisation is not an all-or-none phenomenon. The Gram positive cells may be decolourised by prolonged treatment with the organic solvent. Conversely, inadequate decolourisation may cause all cells to appear Gram positive. The Gram reaction may be related to the permeability of the bacterial cell wall and cytoplasmic membrane to the dye iodine complex, the Gram negative, but not the Gram positive cells, permitting the outflow of the complex during decolourisation. The Gram positive bacteria become Gram negative when the cell wall is damaged.

Gram staining is an essential procedure used in the identification of bacteria and, frequently, is the only method required for studying their morphology. Gram reactivity is of considerable importance as the Gram positive and negative bacteria differ not merely in staining characteristics and in structure, but also in several other properties such as growth requirements, susceptibility to antibiotics and pathogenicity.

The acid fast stain was discovered by Ehrlich, who found that after staining with aniline dyes, tubercle bacilli resist decolourisation with acids. The method, as modified by Ziehl and Neelsen, is in common use now. The smear is stained by a strong solution of carbol fuchsin with the application of heat. It is then decolourised with 20 per cent sulphuric acid and counterstained with a contrasting dye such as methylene blue. The acid fast bacteria retain the fuchsin (red) colour while the others take the counterstain. Acid fastness has been ascribed to the high content and variety of lipids, fatty acids and higher alcohols found in tubercle bacilli. A lipid peculiar to acid fast bacilli, a high molecular weight hydroxy acid wax containing carboxyl groups (mycolic acid), is acid fast in the free state. Acid fastness is not a property of lipids alone, but depends also on the integrity of the cell wall.

Shape of bacteria

Depending on their shape, bacteria are classified into several varieties (Fig. 2.1):

1. *Cocci* (from *kokkos*, meaning berry) are spherical or oval cells.
2. *Bacilli* (from *baculus*, meaning rod) are rod-shaped cells.
3. *Vibrios* are comma shaped, curved rods and derive the name from their characteristic vibratory motility.

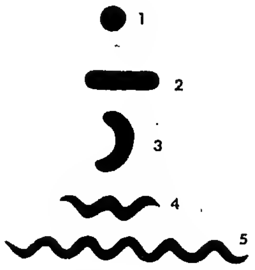


Fig 2.1 Shapes of bacteria 1 coccus 2 bacillus 3. vibrio 4 spirillum 5 spirochaete

4. *Spirilla* are rigid spiral forms.
5. *Spirochaetes* (from *speira*, meaning coil, *chaite*, meaning hair) are flexuous spiral forms.
6. *Actinomycetes* are branching filamentous bacteria, so called because of a fancied resemblance to radiating sun rays when seen in tissue lesions (from *actis*, meaning ray, *mykes*, meaning fungus). The characteristic shape is due to the presence of a rigid cell wall.
7. *Mycoplasmas* are bacteria that are cell wall deficient and hence do not possess a stable morphology. They occur as round or oval bodies and as interlacing filaments. When cell wall synthesis becomes defective, either spontaneously or as a result of drugs such as penicillin, bacteria lose their distinctive shape. Such cells are called *protoplasts*, *spheroplasts* or *L forms*.

Bacteria sometimes show characteristic cellular arrangement or grouping (Fig 2.2). Thus, cocci may be arranged in pairs (*diplococci*), chains (*streptococci*), groups of four (*tetrads*) or

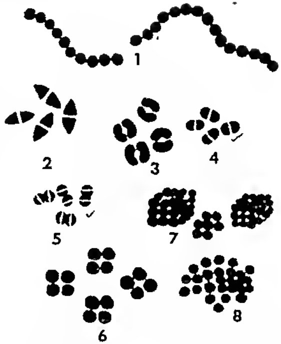


Fig 2.2 Arrangement of cocci: 1 streptococci 2 pneumococci 3. gonococci 4 meningococci 5 *Neisseria catarrhalis* 6. Gaffkya tetragena 7. sarcina 8. staphylococci

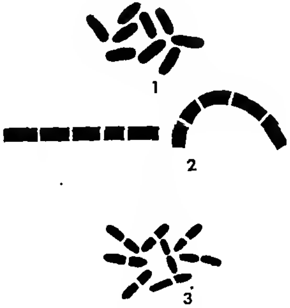


Fig 2.3 Arrangement of bacilli: 1 bacilli in clusters 2. bacilli in chains (*B anthrax*) 3. diplobacilli (*K pneumoniae*)

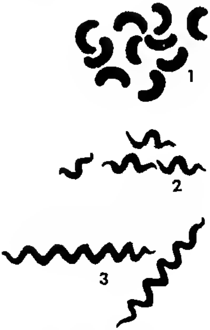


Fig 2.4 Arrangement of curved bacteria: 1. vibrio 2. spirilla 3 spirochaetes

eight (*sarcina*), or as grape-like clusters (*staphylococci*).

Some bacilli also may be arranged in chains (*streptobacilli*). Some other bacilli are arranged at angles to each other, presenting a cuneiform or Chinese letter pattern (*corynebacteria*). The type of cellular arrangement is determined by the plane through which binary fission takes place and by the tendency of the daughter cells to remain attached even after division.

Bacterial anatomy

Fig 2.5 shows the structure an idealised bacterial cell. The outer layer or *cell envelope* consists of two components — a rigid *cell wall* and beneath it a cytoplasmic or plasma membrane. The cell envelope encloses the protoplasm, comprising the *cytoplasm*, cytoplasmic inclusions such as *ribosomes* and *mesosomes*, *granules*, *vacuoles* and the *nuclear body*. Besides these essential components, some bacteria may possess additional structures. The cell may be enclosed in a viscid layer, which may be a loose slime layer, or organised as a *capsule*. Some bacteria carry filamentous appendages protruding from the cell surface — the *flagella* which are organs of locomotion and the *fimbriae* which appear to be organs for adhesion.

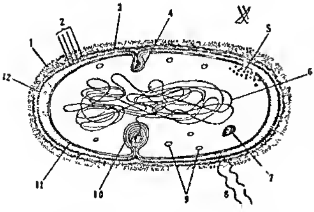


Fig 2.5 Diagram of an *idealised* bacterial cell 1. Capsule 2. Pili 3 Outer membrane 4 Division septum 5 Ribosomes 6 DNA 7. Granular inclusions 8. Flagella 9. Fat globules 10. Mesosome 11 Cytoplasmic membrane 12. Peptidoglycan

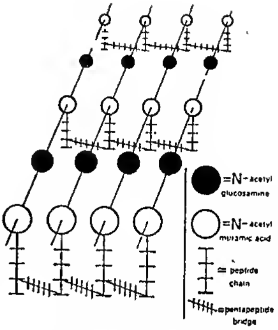


Fig. 2 6 Chemical structure of bacterial cell wall.

The cell wall: The cell wall accounts for the shape of the bacterial cell and confers on it rigidity and ductility. The cell wall cannot be seen by direct light microscopy and does not stain with simple dyes. It may be demonstrated by plasmolysis. When placed in a hypertonic solution, the cytoplasm loses water by osmosis and shrinks, while the cell wall retains the original shape and size (bacterial ghost). The cell wall may also be demonstrated by microdissection, reaction with specific antibody, mechanical rupture of the cell, differential staining procedures or by electron microscopy. Bacterial cell walls are about 10–25 nm in thickness and account for about 20–30 per cent of the dry weight of the cells. Chemically, the cell wall is composed of mucopeptide (*murein*) scaffolding formed by N acetyl glucosamine and N acetyl muramic acid molecules alternating in chains, which are cross linked by peptide chains (Fig 2.6). The interstices of this scaffolding contain other chemicals, varying in the different species. In general, the cell wall of Gram positive bacteria have simpler chemical nature than those of Gram negative bacteria

(Fig. 2.6). The cell wall carries bacterial antigens that are important in virulence and immunity.

The lipopolysaccharides (LPS) present on the cell walls of Gram negative bacteria account for their endotoxic activity and O antigen specificity. They were formerly known as the *Boivin antigen*. The LPS consists of three regions. Region I is the polysaccharide portion determining the O antigen specificity. Region II is the core polysaccharide. Region III is the glycolipid portion (lipid A) and is responsible for the endotoxic activities, viz., pyrogenicity, lethal effect, tissue necrosis, anticomplementary activity, B cell mitogenicity, immunoadjuvant property and antitumour activity.

Cell wall synthesis may be inhibited by many factors. Lysozyme, an enzyme normally present in many tissue fluids, lyses susceptible bacteria by splitting the cell wall mucopeptide linkages. When lysozyme acts on a Gram positive bacterium in a hypertonic solution, a 'protoplast' is formed, consisting of the cytoplasmic membrane and its contents. With Gram negative bacteria the result is a 'spheroplast' which differs from the protoplast in that some cell wall material is retained. Protoplasts and spheroplasts are spherical, regardless of the original shape of the bacterium. Cell wall deficient forms of bacteria may probably have a role in the persistence of certain chronic infections such as pyelonephritis.

Cytoplasmic membrane: The cytoplasmic (plasma) membrane is a thin (5–10 nm) layer lining the inner surface of the cell wall and separating it from the cytoplasm. It acts as a semipermeable membrane controlling the inflow and outflow of metabolites to and from the protoplasm. Passage *through the membrane is not solely a function of* the molecular size of the particles, but depends, in many cases, on the presence in the membrane of specific enzymes (*permeases*). Electron microscopy shows the presence of three layers constituting a 'unit membrane' structure. Chemically, the membrane consists of lipoprotein with small amounts of carbohydrate. Sterols are absent, except in mycoplasma.

Cytoplasm: The bacterial cytoplasm is a colloidal system of a variety of organic and inorganic *solutes in a viscous watery solution. It differs* from eukaryotic cytoplasm in not exhibiting internal mobility (*protoplasmic streaming*) and in the absence of endoplasmic reticulum or mitochondria. The cytoplasm stains uniformly with basic dyes in young cultures, but becomes *increasingly granular with age.* The cytoplasm contains ribosomes, mesosomes, inclusions and vacuoles.

Ribosomes are the centres of protein synthesis. They are slightly smaller than the ribosomes of eukaryotic cells (sedimentation constant 70 S) and are seen integrated in linear strands of mRNA to form polysomes.

Mesosomes (chondroids) are vesicular, convoluted or multilaminated structures formed as invaginations of the plasma membrane into the cytoplasm. They are more prominent in Gram positive bacteria. They are the principal sites of

TABLE 2.2

A comparison of cell walls of Gram positive and Gram negative bacteria

	Gram positive	*Gram negative*
Thickness	Thicker	Thinner
Variety of aminoacids	Few	Several
Aromatic and sulphur-containing aminoacids	Absent	Present
Lipids	Absent or scanty	Present
Teichoic acid	Present	Absent

respiratory enzymes in bacteria and are analogous to the mitochondria of eukaryotes. Mesosomes are often seen in relation to the nuclear body and the site of synthesis of cross wall septa, suggesting that they coordinate nuclear and cytoplasmic division during binary fission.

Intracytoplasmic inclusions may be of various types, the chief of which are volutin, polysaccharide, lipid and crystals. They are characteristic for different species and depend on the age and condition of the culture. *Volutin granules (metachromatic or Babes-Ernst granules)* are highly refractive, strongly basophilic bodies consisting of polymetaphosphate. They appear reddish when stained with polychrome methylene blue or toluidine blue *(metachromasia)*. Special staining techniques such as Albert's or Neisser's demonstrate the granules more clearly. Volutin granules are characteristically present in diphtheria bacilli. Their function is uncertain. They have been considered to represent a reserve of energy and phosphate for cell metabolism, but they are most frequent in cells grown under conditions of nutritional deficiency and tend to disappear when the deficient nutrients are supplied.

Polysaccharide granules may be demonstrated by staining with iodine, and lipid inclusions with fat soluble dyes such as Sudan black. They appear to be storage products. Vacuoles are fluid containing cavities separated from the cytoplasm by a membrane. Their function and significance are uncertain.

Nucleus: The existence of the bacterial nucleus was for long a matter of controversy. They cannot be demonstrated by direct light microscopy. Basic dyes stain the whole bacterial cell intensely and uniformly, without any nucleocytoplasmic differentiation. As bacteria obey the laws of heredity, it was evident that they possessed a functional, if perhaps not a structural, nuclear apparatus. Hence, while some held that bacteria did possess nuclei like higher organisms, others suggested that the whole bacterial cell represented a nucleus.

Bacterial nuclei can be demonstrated by acid or ribonuclease hydrolysis and subsequent staining for nuclear material. They may be seen by electron microscopy. They appear as oval or elongated bodies, generally one per cell. Some cells may possess two or more nuclear bodies due to asynchrony between nuclear and cytoplasmic division.

Bacterial nuclei have no nuclear membrane or nucleolus. The nuclear deoxyribonucleic acid (DNA) does not appear to be associated with basic protein. The genome consists of a single molecule of double-stranded DNA arranged in the form of a circle, which may open under certain conditions to form a long chain, about 1000μ in length. The bacterial chromosome is haploid and replicates by simple fission instead of by mitosis as in higher cells. The differences between the nuclei of bacteria and higher organisms form the main basis for classifying them as prokaryotes and eukaryotes.

Bacteria may possess extranuclear genetic elements consisting of DNA. These cytoplasmic carriers of genetic information are termed *plasmids* or *episomes* (see Chapter 8). Besides being transmitted to daughter cells during binary fission, they may be transferred from one bacterium to another either through conjugation or the agency of bacteriophages. They are not essential for the life of the cell they inhabit, but may confer on it certain properties like toxigenicity and drug resistance which may constitute a survival advantage.

Slime layer and capsule: Many bacteria secrete a viscid material around the cell surface. When this is organised into a sharply defined structure, as in the pneumococcus, it is known as the *capsule*. When it is a loose undemarcated secretion as in leuconostoc, it is called the *slime layer*. Capsules too thin to be seen under the light microscopes are called *microcapsules*. The slime is generally, but not invariably, polysaccharide (e.g., pneumococcus) or polypeptide (e.g., anthrax bacillus) in nature. Some bacteria may have both a capsule and a slime layer (e.g., *Streptococcus salivarius*). Bacteria secreting large amounts of slime pro-

duce mucoid growth on agar, with a stringy consistency when touched with the loop.

Slime has little affinity for basic dyes and is not visible in Gram stained smears. Special capsule staining techniques are available, usually employing copper salts as mordants. Capsules may be readily demonstrated by negative staining in wet films with Indian ink, when they are seen as clear halos around the bacteria, against a black background (Fig. 2.7)

Capsular material is antigenic and may be demonstrated by serological methods. When a suspension of a capsulated bacterium is mixed with its specific anticapsular serum and examined under the microscope, the capsule becomes very prominent and appears 'swollen' due to an increase in its refractivity. This *capsule swelling* or *Quellung reaction*, described by Neufeld (1902), was widely employed for the typing of pneumococci in the pre-sulphonamide days when lobar pneumonia used to be treated with specific anticapsular sera.

Capsules protect bacteria from deleterious agents such as lytic enzymes found in nature. They also contribute to the virulence of pathogenic bacteria by inhibiting phagocytosis. Loss of the capsule by mutation may render the bacterium avirulent. Repeated subcultures *in vitro* lead to the loss of capsule and also of virulence.

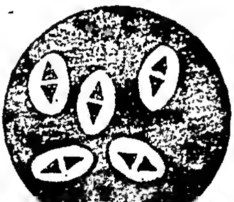


Fig 2.7 Pneumococci negatively stained with Indian ink to show capsule.

Flagella: Motile bacteria, excepting spirochaetes, possess one or more unbranched, long, sinuous filaments called flagella which are the organs of locomotion. Each flagellum consists of three distinct parts, the filament, the hook and the basal body. The filament is external to the cell and connected to the hook at the cell surface. The hook-basal body portion is embedded in the cell envelope. The hook and basal body are antigenically different. Mechanical detachment of the filament does not impair the viability of the cell. The flagella are 3–20 μm long and are of uniform diameter (0.01–0.013 μm) and terminate in a square tip. The wavelength and thickness of the filament are characteristic of each species but some bacteria exhibit biplicity, i.e., have flagella of two different wavelengths (Fig. 2.8). Flagella are made up of a protein (*flagellin*) similar to keratin or myosin. Though flagella of different genera of bacteria have the same chemical composition, they are antigenically different. Flagellar antigens induce specific antibodies in high titres. Flagellar antibodies are not protective, but are useful in serodiagnosis.

The presence or absence of flagella and their number and arrangement are characteristic of different genera of bacteria (Fig. 2.9). Flagella may be arranged all round the cell (*peritrichous*) as in typhoid bacilli, or situated at one or both ends of the cell (*polar*). Polar flagella may be single (*monotrichous*) as in cholera vibrios, in tufts (*lophotrichous*) as in spirilla or with flagella at both poles (*amphitrichous*)

Flagella are only about 0.02μ in thickness and hence beyond the limit of resolution of the light microscope. They may, in some instances, be seen under dark ground illumination. They can be visualised by special staining techniques in which their thickness is increased by mordanting, or by electron microscopy (Fig.2.10). Due to the difficulty of demonstrating flagella directly, their presence is usually inferred by the motility of bacteria. Motility can be observed by noting the spreading type of growth on a semisolid agar medium. Under the microscope, active motility has to be differentiated from the passivie move-

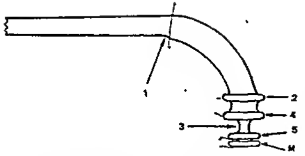


Fig. 2 8 Flagellum and its parts 1. Arrow shows the junction of the hook and filament 2. ring for attachment to outer lipopolysaccharide O antigen-complex membrane of the cell wall 3. rod connecting top and bottom rings 4. ring for its association with the peptidoglycan layer of the cell wall 5. ring located just above cytoplasmic membrane M. ring for attachment to the cytoplasmic membrane.

ments of the cells, either due to air currents or due to Brownian movement. Bacterial motility may range from the slow 'stately' motion of peritrichate bacteria (e.g., *Bacillus*) to the darting movement of polar flagellated vibrios. The cholera vibrio may move as fast as 200μ per second.

Fimbriae: Some Gram negative bacilli carry very fine, hair-like surface appendages called fimbriae or *pili.* They are shorter and thinner than flagella (about 0.5μ long and less than 10 nm thick) and project from the cell surface as straight filaments At least eight morphological types of pili are known, classifiable as either common or sex pili on the basis of function Pili comprise self-aggregating monomers of pilin They originate in the cell membrane Fimbriae can be seen only under the electron microscope. They are unrelated to motility and are found on motile as well as nonmotile cells. They are best developed in freshly isolated strains and in liquid cultures. They tend to disappear following subcultures on solid media.

Fimbriae function as organs of adhesion, helping the cells to adhere firmly to particles of various kinds. This property may serve to anchor the bacteria in nutritionally favourable microenvi- ... Fimbriated bacteria form surface pellicles in liquid media. Many fimbriated cells (e.g., *Escherichia, Klebsiella*) agglutinate red-blood cells of guinea pigs, fowl, horses and pigs strongly, human and sheep cells weakly and ox cells scarcely at all. Haemagglutination provides a simple method for detecting the presence of such fimbriae. The haemagglutination is specifically inhibited by D mannose.

Fimbriae are antigenic. As members of different genera may possess the same fimbrial antigen, it is necessary to ensure that the bacterial antigens employed for serological tests and preparation of antisera are devoid of fimbriae.

A special type of fimbria is the sex pilus. These are longer and fewer in number than other fimbriae. They are found on 'male' bacteria and help in the attachment of those cells to 'female' bacteria, forming hollow 'conjugation-tubes' through which, it is assumed, genetic material is transferred from the donor to the recipient cell. Pili are classified into different types (e.g., F, I) based on susceptibility to specific bacteriophages.

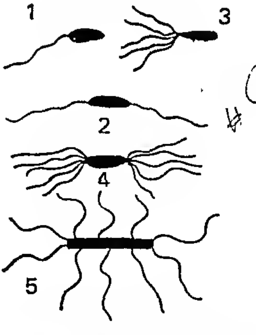


Fig 2 9 Types of flagellar arrangement 1. single polar flagellum 2. single flagellum at each pole 3. tuft of flagella at one pole 4 tufts of flagella at both poles 5 peritrichous

Spore: Some bacteria, particularly members of the genera *Bacillus* and *Clostridium*, have the ability to form highly resistant resting stages called spores. Each bacterium forms one spore, which on germination forms a single vegetative cell. Sporulation in bacteria, therefore, is not a method of reproduction. As bacterial spores are formed inside the parent cell, they are called endospores.

While the exact stimulus for sporulation is not known, it occurs after a period of vegetative growth and is presumed to be related to the depletion of exogenous nutrients. Sporulation is initiated by the appearance of a clear area, usually near one end of the cell, which gradually becomes more opaque to form the 'forespore'. The fully developed spore has at its core the nuclear body, surrounded by the spore wall, a delicate membrane from which the cell wall of the future vegetative bacterium will develop. Outside this is the thick spore cortex, which in turn is enclosed by a multilayered tough spore coat. Some spores have an additional outer covering called exosporium, which may have distinctive ridges and grooves (Fig. 2.11). New antigens appear on sporulation.

Young spores are seen attached to the parent cell. The shape and position of the spore and its size relative to the parent cell are species characteristics. Spores may be central (equatorial), terminal or subterminal. They may be oval or spherical. They may or may not distend the bacillary body (Fig. 2.12).

Bacterial spores constitute some of the most resistant forms of life. They may remain viable for centuries. They are extremely resistant to desiccation and relatively so to chemicals and heat. Though some spores may resist boiling for prolonged periods, spores of all medically important bacteria are destroyed by autoclaving at 120°C for 15 minutes. Methods of disinfection and sterilisation should ensure destruction of spores also. Sporulation helps bacteria to survive for long periods under unfavourable environments.

When transferred to conditions conducive to vegetative growth, spores germinate. The spore loses its refractility, and swells. The spore wall is

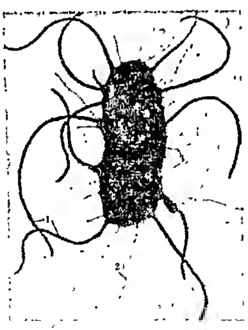


Fig 2.10 Electron micrograph of *E. coli*. 1. Flagella 2. F. pili 3. ordinary pili or fimbriae

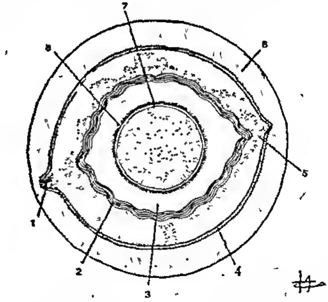
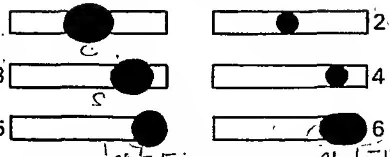


Fig. 2.11 Diagrammatic representation of a bacterial spore. 1. germinal groove 2. outer cortical layer 3. cortex 4. internal spore coat 5. subcoat material 6 outer spore coat 7. cytoplasmic membrane 8. cell wall primordium

Ba. anthracis

Cl. septicum, Cl. sporogenes



Cl. tetani Cl. tertium.

Fig 2 12 Types of bacterial spores. 1. central, bulging, 2. central, not bulging, 3 subterminal, bulging, 4. subterminal, not bulging, 5 terminal, spherical, 6. terminal, oval

shed and the germ cell appears by rupturing the spore coat. The germ cell elongates to form the vegetative bacterium.

Spores may be seen in unstained preparation as refractile bodies. The forespore stains intensely, but once the spore envelope is laid down, the spore does not stain readily. Spores appear as unstained areas in Gram stained preparations, but being more acid fast than the vegetative cells, they can be stained by a modification of the Ziehl-Neelsen technique

Pleomorphism and involution forms: Some species of bacteria exhibit great variation in the shape and size of individual cells. This is known as pleomorphism. Certain species (e.g., plague bacillus, gonococcus) show swollen and aberrant forms in ageing cultures, especially in the presence of high salt concentration. These, are known as involution forms. Many of the cells may be nonviable. Pleomorphism and involution forms are often due to defective cell wall synthesis. Involution forms may also develop due to the activity of autolytic enzymes.

L forms. Kleineberger-Nobel, studying cultures of *Streptobacillus moniliformis*, observed swollen cells and other aberrant morphological forms and named them L forms, after the Lister Institute, London, where the observation was made. forms are seen in several species of bacteria, loping either spontaneously or in the presence of penicillin or other agents that interfere with cell wall synthesis. L forms may be unstable in that the morphological abnormality is maintained only in the presence of penicillin and other inducing agents, or stable, when the aberrant form becomes the permanent feature of the strain and is retained in serial subcultures.

L forms resemble mycoplasmas in several ways, including morphology, type of growth on agar and filterability. It is possible that mycoplasmas represent stable L forms of as yet unidentified parent bacteria. L forms of bacteria have been isolated from patients suffering from chronic urinary and suppurative infection but their role in the causation and maintenance of disease remains to be clarified.

Growth and multiplication of bacteria

Bacteria divide by binary fission. When a bacterial cell reaches a certain size, it divides to form two daughter cells. Nuclear division precedes cell division and, therefore, in a growing population, many cells carrying two nuclear bodies can be seen. The cell divides by a constrictive or pinching process, or by the ingrowth of a transverse septum across the cell. In some species, the daughter cells may remain partially attached after division.

The interval of time between two cell divisions, or the time required for a bacterium to give rise to two daughter cells under optimum conditions, is known as the *generation time* or *population doubling time.* In coliform bacilli and many other

medically important bacteria, the generation time is about 20 minutes. Some bacteria are slow-growing; the generation time in tubercle bacilli is about 20 hours and in lepra bacilli as long as about 20 days. As bacteria reproduce so rapidly and by geometric progression, a single bacterial cell can theoretically give rise in 24 hours to 10^{21} progeny, with a mass of approximately 4000 tons! In actual practice, when bacteria are grown in a vessel of liquid medium (batch culture), multiplication is arrested after a few cell divisions due to depletion of nutrients or accumulation of toxic products. By the use of special devices for replenishing nutrients and removing bacterial cells (chemostat or turbidistat), it is possible to maintain *continuous culture* of bacteria for industrial or research purposes. When pathogenic bacteria multiply in host tissues, the situation may be intermediate between a batch culture and continuous culture; the source of nutrients may be inexhaustible, but the parasite has to contend with the defence mechanisms of the body. Bacteria growing on solid media form *colonies*. Each colony represents a clone of cells derived from a single parent cell. In liquid media, growth is diffuse.

Bacterial growth may be considered at two levels, increase in the size of the individual cell and increase in the number of cells. The former is ordinarily limited and when the critical size is reached, the cell divides, except when cell division is inhibited by substances like penicillin or acriflavine or by growth in Mg^{++} deficient media. Growth in numbers can be studied by bacterial counts. Two types of bacterial counts can be made, total count and viable count.

The *total count* gives the total number of cells in the sample, irrespective of whether they are living or not. It can be obtained by

1. direct counting under the microscope using counting chambers;
2. counting in an electronic device as in the Coulter counter;
3. direct counting using stained smears prepared by spreading a known volume of the culture over a measured area of a slide;
4. comparing relative numbers in smears of the culture mixed with known numbers of other cells;
4. by opacity measurements using an absorptiometer or nephalometer;
6. by separating the cells by centrifugation or filtration and measuring their wet or dry weight: and
7. chemical assay of cell components such as nitrogen.

The *viable count* measures the number of living cells, i.e., cells capable of multiplication. Viable counts are obtained by dilution or plating methods. In the dilution method, the suspension is diluted to extinction, i.e., to a point beyond which unit quantities do not yield growth when inoculated into suitable liquid media. Several tubes are inoculated with varying dilutions and the viable count calculated statistically from the number of tubes showing growth. The method does not give accurate values, but is used widely in water bacteriology for estimation of the 'presumptive coliform count' in drinking water. In the plating method, appropriate dilutions are inoculated on solid media, either on the surface of plates or as pour plates. The number of colonies that develop after incubation gives an estimate of the viable count. The method commonly employed is that described by Miles and Misra (1938) in which serial dilutions are dropped on the surface of dried plates and colony counts obtained.

Bacterial growth curve

When a bacterium is seeded into a suitable liquid medium and incubated, its growth follows a definite course. If bacterial counts are made at intervals after inoculation and plotted in relation to time, a growth curve is obtained (Fig. 2.13).

The curve shows the following phases:

1. *Lag phase:* Immediately following the seeding of a culture medium, there is no appreciable increase in numbers, though there may be an

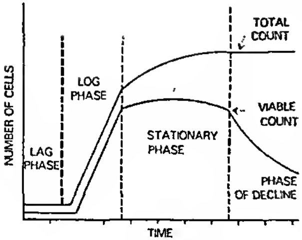


Fig. 2.13 Bacterial growth curve. The viable count shows lag, log, stationary and decline phases. In the total count, the phase of decline is not evident

increase in the size of the cells. This initial period is the time required for adaptation to the new environment, during which the necessary enzymes and metabolic intermediates are built up in adequate quantities for multiplication to proceed. The duration of the lag phase varies with the species, size of inoculum, nature of culture medium and environmental factors such as temperature.

2. *Log (logarithmic) or exponential phase:* Following the lag phase, the cells start dividing and their numbers increase exponentially or by geometric progression with time. If the logarithm of the viable count is plotted against time, a straight line will be obtained.

3. *Stationary phase:* After a varying period of exponential growth, cell division comes to a halt due to depletion of nutrients and accumulation of toxic products. The number of progeny cells formed is just enough to replace the number of cells that die. The viable count remains stationary as an equilibrium exists between the dying cells and the new-formed cells.

4. *Phase of decline:* This is the phase when the population decreases due to the death of cells. Besides nutritional exhaustion and toxic accumulation, cell death may be caused by autolytic enzymes also.

When the total count is plotted, it parallels the viable count up to the stationary phase, but it continues steadily without any phase of decline. With autolytic bacteria, even the total count shows a phase of decline.

The various stages of the growth curve are associated with morphological and physiological alterations of the cells. Bacteria have the maximum cell size towards the end of the lag phase. In the log phase, cells are smaller and stain uniformly. In the stationary phase, cells frequently are Gram variable and show irregular staining due to the presence of intracellular storage granules. Sporulation occurs at this stage. Also, many bacteria produce secondary metabolic products such as exotoxins and antibiotics. Involution forms are common in the phase of decline.

Bacterial nutrition

The bacterial cell has the same general chemical pattern as the cells of higher organisms. The principal constituent of bacterial cells is water, which represents about 80 per cent of the total weight. Proteins, polysaccharides, lipids, nucleic acids, mucopeptides and low molecular weight compounds make up the rest. Bacterial metabolism also is closely similar to the metabolism of the higher organisms, exemplifying the 'unity of biochemistry'. There are, however, some differences which are exploited in selective toxicity and chemotherapy.

For growth and multiplication of bacteria, the minimum nutritional requirements are water, a source of carbon, a source of nitrogen and some inorganic salts. Water is the vehicle for the entry of all nutrients into the cells and for the elimination of all waste products. It participates in the metabolic reactions and also forms an integral part of the protoplasm.

Bacteria can be classified nutritionally, based on their energy requirements and on their ability

to synthesise essential metabolites. Bacteria which derive their energy from sunlight are called *phototrophs* and those that obtain energy from chemical reactions are called *chemotrophs*. Bacteria that can synthesise all their organic compounds are called *autotrophs*. Those that are unable to synthesise their own metabolites and depend on preformed organic compounds are called *heterotrophs*. Autotrophs are able to utilise atmospheric carbon dioxide and nitrogen. They are capable of independent existence in water and soil and are of no medical importance, though they are of vital concern in agriculture and the maintenance of soil fertility. Heterotrophic bacteria are unable to grow with carbon dioxide as the sole source of carbon. The nutritional requirements of heterotrophs vary widely. Some may require only a single organic substance such as glucose, while others may need a large number of different compounds such as aminoacids, nucleotides, lipids, carbohydrates and coenzymes.

Bacteria require a supply of inorganic salts, particularly the anions phosphate and sulphate, and the cations sodium, potassium, magnesium, iron, manganese and calcium. These are normally present in the natural environments where bacteria live, but will have to be supplied in culture media. Some ions such as cobalt may be needed in trace amounts.

Some bacteria require for growth certain organic compounds in minute quantities. These are known as growth factors or bacterial vitamins. Growth factors may be 'essential' when growth does not occur in their absence, or 'accessory' when they enhance growth, without being absolutely necessary for it. In many cases, bacterial vitamins are identical with the vitamins necessary for mammalian nutrition, particularly those belonging to the B group — thiamine, riboflavine, nicotinic acid, pyridoxine, folic acid and vitamine B_{12}

If a microorganism requiring an essential growth factor is inoculated into a medium containing an excess of all other nutrients, its growth will be proportional to the amount of the limiting substance added. Within a certain range, the concentration of the growth factor will bear a linear relationship with the amount of growth of the organism. This is the principle of microbiological assays, which provide a very sensitive and specific method for estimation of many aminoacids and vitamins, as in the determination of vitamin B_{12} using *Lactobacillus leichmannii*.

Oxygen requirement and metabolism

Depending on the influence of oxygen on growth and viability, bacteria are divided into aerobes and anaerobes. Aerobic bacteria require oxygen for growth. They may be obligate aerobes like the cholera vibrio, which will grow only in the presence of oxygen, or facultative anaerobes which are ordinarily aerobic, but can grow also in the absence of oxygen, though less abundantly. Most bacteria of medical importance are facultative anaerobes. Anaerobic bacteria, such as clostridia, grow in the absence of oxygen and the obligate anaerobes may even die on exposure to oxygen. Microaerophilic bacteria are those that grow best in the presence of a low oxygen tension.

The reason for the apparent toxicity of oxygen for anaerobic bacteria is not well understood. It has been suggested that in the presence of oxygen, hydrogen peroxide and other toxic peroxides accumulate. The enzyme catalase which splits hydrogen peroxide is present in most aerobic bacteria, but is absent in the anaerobes. Another reason might be that obligate anaerobes possess essential enzymes that are active only in the reduced state.

The influence of free oxygen is related to the metabolic character of the bacterium. Aerobic bacteria obtain their energy and intermediates only through oxidation involving oxygen as the ultimate hydrogen acceptor, while the anaerobes use hydrogen acceptors other than oxygen. Facultative anaerobes may act in both ways. In the case of aerobes, where the ultimate electron acceptor is atmospheric oxygen (aerobic respiration), the carbon and energy source may be completely oxidised to carbon dioxide and water.

Energy is provided by the production of energy-rich phosphate bonds and the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This process is known as oxidative phosphorylation. Anaerobic bacteria use as electron acceptors compounds such as nitrates or sulphates, instead of oxygen (anaerobic respiration). A more common process in anaerobic metabolism may be a series of oxidoreductions in which the carbon and energy source acts both as the electron donor and electron acceptor. This process is known as fermentation and leads to the formation of several organic end-products such as acids and alcohols, as well as of gas (carbon dioxide and hydrogen). During the process of fermentation, energy-rich phosphate bonds are produced by the introduction of organic phosphate into intermediate metabolites. This process is known as substrate-level phosphorylation. The energy-rich phosphate groups so formed are used for conversion of ADP to ATP

In determining the growth of aerobic and anaerobic bacteria, what is more important, than the presence or absence of oxygen as such, is the state of oxidation of the environment. The oxidising or reducing condition of a system is indicated by the net readiness of all the components in that system to take up, or part with electrons. This is known as the oxidation-reduction (redox) potential of the system. The redox potential of a medium is best estimated by measuring the electrical potential difference set up between the medium and an 'unattackable' electrode immersed in it This electrode potential (*Eh*) can be measured in millivolts. The more oxidised the system, the higher is the potential. A simpler, though less accurate, method of measuring the redox potential is the use of oxidation-reduction indicators such as methylene blue, and noting the colour change.

Carbon dioxide

All bacteria require small amounts of carbon dioxide for growth. This requirement is usually met by the carbon dioxide present in the atmosphere, or produced endogenously by cellular metabolism. Some bacteria like *Brucella abortus* require much higher levels of carbon dioxide (5–10 per cent) for growth, especially on fresh isolation.

Temperature

Bacteria vary in their requirements of temperature for growth For each species, there is a 'temperature range', and growth does not occur above the maximum or below the minimum of the range. The temperature at which growth occurs best is known as the 'optimum temperature', which in the case of most pathogenic bacteria is 37°C. Bacteria which grow best at temperatures of 25–40°C are called *mesophilic*. All parasities of warm-blooded animals are mesophilic. Within the group of mesophilic bacteria, some like *Pseudomonas aeruginosa* have a wider range (5–43°C) while others like the gonococcus have a restricted range (30–39°C).

Psychrophilic bacteria are those that grow best at temperatures below 20°C, some of them even growing at temperatures as low as −7°C They are soil and water saprophytes and, though not of direct medical importance, may cause spoilage of refrigerated food. Another group of non-pathogenic bacteria, the *thermophiles*, grow best at high temperatures, 55–80°C. They may cause spoilage of underprocessed canned foods. Some thermophiles (e.g , *Bacillus stearothermophilus*) form spores that are exceptionally thermoresistant. Extremely thermophilic bacteria have been identified which can grow at temperatures as high as 250°C.

Bacteria also differ in the effect of temperature on viability. Heat is an important method for the destruction of microorganisms (sterilisation). moist heat causing coagulation and denaturation of proteins and dry heat causing oxidation and charring Moist heat is more lethal than dry heat. The lowest temperature that kills a bacterium under standard conditions in a given time is known as the *thermal death point*. Under moist

conditions most vegetative mesophilic bacteria have a thermal death point between 50° and 65°C and most spores between 100° and 120°C.

At low temperatures some species die rapidly, but most survive well. Storage in the refrigerator (3–5°C) or the deep freeze cabinet (−30° to −70°C) is used for preservation of cultures. Rapid freezing as with solid carbon dioxide or the use of a stabiliser such as glycerol minimises the death of cells on freezing.

Moisture and drying

Water is an essential ingredient of bacterial protoplasm and hence drying is lethal to cells. However, the effect of drying varies in different species. Some delicate bacteria like *Treponema pallidum* are highly sensitive, while others like staphylococci withstand drying for months. Spores are particularly resistant to desiccation and may survive in the dry state for several decades. Drying in vacuum, in the cold (freeze drying or lyophilisation) is a method for the preservation of bacteria, viruses and many labile biological materials.

H-ion concentration

Bacteria are sensitive to variations in pH. Each species has a pH range, above or below which it does not survive, and an optimum pH at which it grows best. The majority of pathogenic bacteria grow best at neutral or slightly alkaline reaction (pH 7.2–7.6). Some acidophilic bacteria such as lactobacilli grow under acid conditions. Others, such as the cholera vibrio, are very sensitive to acid, but tolerate high degrees of alkalinity. Strong soluions of acid or alkali (5% hydrochloric acid or sodium hydroxide) readily kill most bacteria, though mycobacteria are exceptionally resistant to them.

Light

Bacteria (except phototrophic species) grow well in the dark. They are sensitive to ultraviolet light and other radiations. Cultures die if exposed to sunlight. Exposure to light may influence pigment production. Photochromogenic mycobacteria form pigment only on exposure to light and not when incubated in the dark.

Osmotic effect

Bacteria are more tolerant to osmotic variation than most other cells, due to the mechanical strength of their cell walls. Sudden exposure to hypertonic solutions may cause *plasmolysis* — osmotic withdrawal of water and shrinkage of protoplasm. This occurs more readily in Gram negative than in Gram positive bacteria. Sudden transfer from a concentrated solution to distilled water may cause *plasmoptysis* — excessive osmotic imbibition leading to swelling and rupture of the cell.

Mechanical and sonic stress

Though bacteria have tough cell walls, they may be ruptured by mechanical stresses such as grinding or vigorous shaking with glass beads. They may also be disintegrated by exposure to ultrasonic vibration.

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3 Sterilisation and Disinfection

Microorganisms are ubiquitous. Since they cause contamination, infection and decay, it becomes necessary to remove or destroy them from materials or from areas. This is the object of sterilisation. The process of sterilisation finds application in microbiology for prevention of contamination by extraneous organisms, in surgery for maintenance of asepsis, in food and drug manufacture for ensuring safety from contaminating organisms and in many other situations. The methods of sterilisation employed would depend on the purpose for which sterilisation is carried out, the material which has to be sterilised and the nature of the microorganisms that are to be removed or destroyed.

Sterilisation is defined as the process by which an article, surface or medium is freed of all microorganisms either in the vegetative or spore state. *Disinfection* means the destruction of all pathogenic organisms, or organisms capable of giving rise to infection. The term *antisepsis* is used to indicate the prevention of infection, usually by inhibiting the growth of bacteria. Chemical disinfectants which can be safely applied to skin or mucous membrane surfaces and are used to prevent infection by inhibiting the growth of bacteria are called antiseptics. Bactericidal agents are those which are able to kill bacteria. Bacteriostatic agents only prevent the multiplication of bacteria and they may remain alive. One and the same chemical, bactericidal at a particular concentration, may be only bacteriostatic at a higher dilution

The various agents used in sterilisation can be classified as follows:

A. Physical agents

1. Sunlight. 2. Drying. 3. Dry heat: flaming, incineration, hot air. 4. Moist heat: pasteurisation, boiling, steam under normal pressure, steam under pressure. 5. Filtration: candles, asbestos pads, membranes. 6. Radiation. 7. Ultrasonic and sonic vibrations.

B. Chemicals

1. Alcohols: ethyl, isopropyl, trichlorobutanol. 2. Aldehydes: Formaldehyde, Glutaraldehyde 3. Dyes, 4. Halogens, 5. Phenols, 6. Surface-active agents, 7. Metallic salts, 8. Gases: Ethylene oxide, Formaldehyde, Betapropiolactone.

Sunlight

Sunlight possesses appreciable bactericidal activity and plays an important role in the spontaneous sterilisation that occurs under natural conditions. The action is primarily due to its content of ultraviolet rays, most of which, however, are screened out by glass and the presence of ozone in the outer regions of the atmosphere. Under natural conditions, its sterilising power varies according to circumstances. Direct sunlight, as in the tropical countrysides, when it is not filtered off by impurities in the atmosphere, has an active germicidal effect due to the combined effect of the ultraviolet and heat rays. Semple and Grieg showed that, in India, typhoid bacilli exposed to the sun on pieces of white drill cloth were killed in

hours, whereas controls kept in the dark
still alive after six days. Bacteria suspended ater are readily destroyed by exposure to sun-. This is one of the natural methods of sterili- n in cases of water in tanks, rivers and lakes.

ing

sture is essential for the growth of bacteria. r-fifths by weight of the bacterial cell consists ater. Drying in air has, therefore, a deleteri- effect on many bacteria. Susceptibility to dry- varies with different bacteria and also on the litions under which they are exposed to dry- *i.e., on cloth, furniture, surrounded by* anic matter, etc. This method is unreliable is only of theoretical interest. Spores are ffected by drying.

it

it is the most reliable method of sterilisation should be the method of choice unless con- ndicated. Materials damageable by heat can sterilised at lower temperatures, for longer iods or by repeated cycles. The factors uencing sterilisation by heat are:

ature of heat — whether dry heat or moist eat,

emperature and time,

he number of microorganisms present,

he characteristics of the organisms, such as pecies, strain, sporing capacity, and

he type of material from which the organisms ave to be eradicated.

The mechanism by which organisms are troyed by moist heat is different from that of heat. The killing effect of dry heat is due to tein denaturation, oxidative damage and the ic effect of elevated levels of electrolytes. The al effect of moist heat is due to the denatura- and coagulation of protein. The advantage of am lies in the latent heat liberated when it con- ses on a cooler surface, raising the tempera- e of that surface. In the case of the spore, am condenses on it, increases its water content with ultimate hydrolysis and breakdown of the bacterial protein. In a completely moisture-free atmosphere, bacteria, like many proteins, are more resistant to heat. They are killed when oxidation of the cell constituents occurs and this requires much higher temperatures than that needed for coagulation of proteins.

The time required for sterilisation is inversely proportional to the temperature of exposure. This can be expressed as thermal death time which is the minimum time required to kill a suspension of organisms at a predetermined temperature in a specified environment. The sterilisation time is directly related to the number of organisms in the suspension, whether spores are present and also the nature of the spores, the strain and characteristics of the organism. The time taken for reaching the required level of temperature should always be kept in mind, since recommended minimum sterilising times do not include that. The nature of the material in which the organisms are heated affects the rate of killing. A high content of organic substances generally tends to protect the spores and vegetative organisms against the lethal action of heat. Proteins, nucleic acids, starch, gelatin, sugar, fats and oils, all increase the time required, particularly fats and oils. The last two prevent access of moisture to the organisms. The presence of disinfectants facilitates the destruction of the organisms. The destruction of spores is facilitated in acid or alkaline pH.

Dry heat

Flaming: Inoculating loops or wires, points of forceps and searing spatulas are held in a bunsen flame till they become red hot, for sterilising them. If the loops contains infective proteinaceous material they should be first dipped in chemical disinfectants before flaming to prevent spattering.

Scalpels, needles, mouths of culture tubes, glass slides, cover slips, etc. could be passed a few times through the bunsen flame without allowing

them to become red hot. The bacteria get destroyed. Needles, basins and scalpels are sometimes immersed in methylated spirit and the spirit burnt off them. This is, however, unsatisfactory.

Incineration: This is an excellent method for rapidly destroying materials such as soiled dressings, animal carcasses, bedding and pathological material. Plastics such as PVC and polythene can be dealt with similarly but polystyrene materials emit clouds of dense black smoke and hence should be autoclaved in appropriate containers.

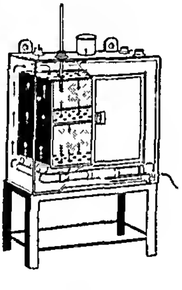

Fig 3.1 Hot air oven

Hot air oven: This is the most widely used method of sterilisation by dry heat. A holding period of 160°C for one hour is used. It is used to sterilise glassware, forceps, scissors, scalpels, all-glass syringes, swabs, some pharmaceutical products such as liquid paraffin, sulphonamides, dusting powder, fats, greases, etc. Hot air is a bad conductor of heat and its penetrating power is low. The oven is usually heated by electricity, with heating elements in the wall of the chamber and it must be fitted with a fan to ensure even distribution of air and elimination of air pockets. (Fig 3.1). It should not be overloaded. The material should be arranged in a manner which allows free circulation of air in between. Glassware should be perfectly dry before being placed in the oven. Test tubes, flasks, etc. should be plugged with cottonwool. Other glassware such as Petri dishes and pipettes should be wrapped in kraft paper. Rubber materials — except silicone rubber — will not stand the temperature. At 180°C cottonwool plugs may get charred. For cutting instruments such as those used in ophthalmic surgery, a sterilising time of two hours at 150°C is recommended. The British Pharmacopoeia recommends a holding time of one hour at 150°C for oils, glycerol and dusting powder. The oven must be allowed to cool slowly for about two hours before the door is opened, since the glassware may get cracked by sudden or uneven cooling

Sterilisation control: The spores of a non-toxigenic strain of *Clostridium tetani* are used as a microbiological test of dry heat efficiency. Paper strips impregnated with 10^6 spores are placed in envelopes and inserted into suitable packs. After sterilisation is over, the strips are removed and inoculated into thioglycollate or cooked meat media and incubated for sterility test under strict anaerobic conditions for five days at 37°C.

A Browne's tube (green spot) is available for dry heat and is convenient for routine use. After proper sterilisation a green colour is produced (after 60 minutes at 160°C or 115 minutes at 150°C).

Thermocouples may also be used periodically.

Moist heat

Temperatures below 100°C: For pasteurisation of milk: The temperature employed is either 63°C for 30 minutes (the holder method) or 72°C for 15–20 seconds (the flash process) followed by cooling quickly to 13°C or lower. By these processes all nonsporing pathogens such as mycobacteria, brucellae and salmonellae are destroyed.

iella burnetii is relatively heat resistant and survive the holder method.

'accines of nonsporing bacteria are heat inactivated in special vaccine baths at 60°C for one r. Serum or body fluids containing coagulable teins can be sterilised by heating for one hour 5°C in a water bath on several successive days.

Media such as Lowenstein-Jensen's and Loef-'s serum are rendered sterile by heating at 80–C for half an hour on three successive days in nspissator.

Though practically all mesophilic nonsporing teria are killed by exposure to moist heat at C for 30 minutes, *Staphylococcus aureus* and *ptococcus faecalis* require 60 minutes. A perature of 80°C for 5–10 minutes destroys vegetative forms of all bacteria, yeasts and ulds. Among the most heat-resistant cells are spores of *Clostridium botulinum* which uire 120°C for four minutes, or 100°C for 330 nutes for their destruction. Amongst viruses, lomyelitis virus requires 60°C for 30 minutes l hepatitis virus in serum 60°C for 10 hours. ne bacteriophages require a temperature ge of 65–80°C for their destruction.

Clothing, bed clothes, eating utensils and some ipment may be disinfected by washing in ter at ~~70–80°C for several minutes.~~

Cystoscopes, specula and apparatus that may damaged by boiling can be disinfected in a h at 75°C for 10 minutes.

nperature at 100°C: Boiling: Vegetative bacia are killed almost immediately at 90–100°C, sporing bacteria require considerable periods boiling. Boiling is *not* recommended for the rilisation of instruments used for surgical prolures and should be regarded only as a means disinfection. Nothing short of autoclaving at h pressure can destroy spores and ensure rilisation. Hard water should not be used. rilisation may be promoted by the addition of sodium bicarbonate to the water.

In cases where boiling is considered adequate, material should be immersed in the water and iled for a period of 10–30 minutes. The lid of the steriliser should not be opened during the period.

Steam at atmospheric pressure (100°C): An atmosphere of free steam is used to sterilise culture media which may decompose if subjected to higher temperatures. A Koch or Arnold steamer is usually used. (Laboratory autoclaves can also be used for this purpose.) This is a cheap method. The container and medium are simultaneously sterilised, evaporation from the medium is prevented and the apparatus requires little or no attention.

The usual steamer consists of a tinned copper cabinet with the walls suitably lagged. The lid is conical, enabling drainage of condensed steam, and a perforated tray fitted above the water level ensures that the material placed on it is surrounded by steam (Fig. 3.2). One exposure of ninety minutes usually ensures complete sterilisation but for media containing sugars or gelatin an

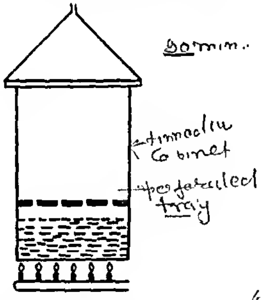

Fig. 3.2 Steamer

exposure of 100°C for 20 minutes on three successive days is used. This is known as ~~tyndallisation~~ or *intermittent sterilisation*. The principle is that the first exposure kills all vegetative bacteria, and spores present, being in a favourable medium,

will germinate and be killed on the subsequent occasions. Though this is generally adequate, it may fail in the case of spores of certain anaerobes and thermophiles.

Steam under pressure: The principle of the autoclave or steam steriliser is that water boils when its vapour pressure equals that of the surrounding atmosphere. Hence when pressure inside a closed vessel increases, the temperature at which water boils also increases. Saturated steam has greater penetrative power. When steam comes into contact with a cooler surface it condenses to water and gives up its latent heat to that surface (1600 ml of steam at 100°C and at atmospheric pressure condenses into one ml of water at 100°C and liberates 518 calories of heat). The large reduction in volume sucks in more steam to the area and the process continues till the temperature of that surface is raised to that of the steam. The condensed water ensures moist conditions for killing the microbes present

Sterilisation by steam under pressure is carried out at temperatures between 108°C and 147°C. By using the appropriate temperature and time, a variety of materials such as dressings, instruments, laboratoryware, media and pharmaceutical products can be sterilised. Aqueous solutions are sterilised between 108°C and 126°C. Heat is conducted through the walls of the sealed containers until the temperature of the fluid inside is in equilibrium with the steam outside.

Several types of steam sterilisers are in use

1. laboratory autoclaves,
2. hospital dressing sterilisers,
3. bowl and instrument sterilisers, and
4. rapid cooling sterilisers.

In its simplest form, the laboratory autoclave consists of a vertical or horizontal cylinder of gunmetal or stainless steel, in a supporting sheet-iron case. The lid or door is fastened by screw clamps and made airtight by an asbestos washer. The autoclave has on its lid or upper side a discharge tap for air and steam, a pressure gauge and a safety valve that can be set to blow off at any desired pressure. Heating is done by gas or electricity (Fig. 3.3).

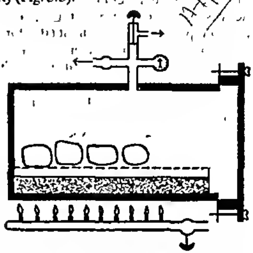

Fig 3.3 A simple autoclave

Sufficient water is put in the cylinder, the material to be sterilised is placed on the tray, and heating is started. The lid is screwed tight with the discharge tap open. The safety valve is adjusted to the required pressure. The steam air mixture is allowed to escape freely till all air has been displaced. This can be tested by allowing the escaping steam into a pail of water through a rubber tubing. When no more air bubbles come out in the pail, the discharge tap is closed. The steam pressure rises inside and when it reaches the desired set level, the safety valve opens and the excess steam escapes. From this point, the holding period is calculated. When the holding period is over, the heater is turned off and the autoclave allowed to cool till the pressure gauge indicates that the inside is at atmospheric pressure. The discharge tap is opened slowly and air allowed to enter the autoclave. If the tap is opened when the pressure inside is high, liquid media will tend to boil violently and spill from their containers and sometimes an explosion may occur. If not opened till pressure inside has fallen below atmospheric pressure, an excessive amount of water will be evaporated and lost from the media.

The defects in this type of autoclave are:

1. The method of air discharge is inefficient, and it is difficult to decide when the discharge is

complete. If air is not completely removed, the desired temperature will not be attained.

2. There is no facility for drying the load after sterilisation and before taking it out.

The domestic pressure cooker serves as a miniature autoclave and may be used for sterilisation of small articles in clinics and similar establishments.

A wide variety of autoclaves have been manufactured incorporating various devices for overcoming these defects and other difficulties in working. The details of their construction and the methods of their operation are outside the purview of this book and readers are referred to the exhaustive manuals on the subject.

Sterilisation control: For determining the efficacy of moist-heat sterilisation, spores of *Bacillus stearothermophilus* are used as the test organism. This is a thermophilic organism with an optimum growth temperature of 55–60°C and its spores require an exposure of 12 minutes at 121°C to be killed. Paper strips impregnated with 10^6 spores are dried at room temperature and placed in paper envelopes. These envelopes are inserted in different parts of a load and after sterilising, the strips are inoculated into a suitable recovering medium and incubated for sterility test at 55°C for five days.

Chemical indicators, autoclave tapes and thermocouples are also used instead.

Filtration

This is the method used to rid heat labile liquids of microorganisms. This is useful for antibiotic solutions, sera and carbohydrate solutions used in the preparation of culture media. By this technique, we can obtain bacteria-free filtrates of toxins and bacteriophages. This method is also useful when we want to separate microorganisms which are scanty in fluids and study them. The filter would retain the organisms and the filter disc could be cultured. There are different types of filters, each type having its own application. What are ordinarily referred to as 'sterilising grades' do not keep back mycoplasma and viruses. Thus, the serum 'sterilised' by Seitz filtration is not safe for clinical use. The filtration is done under carefully controlled positive or negative pressure.

The various types of filters in use in microbiology are:

1. Earthenware candles—Berkefeld, Chamberland, Mandler.
2. Asbestos disc filters, e.g., Seitz.
3. Sintered glass filters.
4. Collodion or membrane filters.

TABLE 3.1

Times and temperatures for heat sterilisation
(Recommendations of the Medical Research Council)

Method	Temperature	Holding time in minutes
Autoclave	121	15
	126	10
	134	3
Hot air oven	160	45
	170	18
	180	7.5
	190	1.5

Candle type filters: Berkefeld filters are made from kieselguhr, a fossilised diatomaceous earth found in Germany. They are available in three grades of porosity, viz., V (viel) the coarsest, W (wenig) the finest, and N (normal) intermediate. Of these, V is the usual one employed and it should not pass *Serratia marcescens,* i.e., 0.75 μ or less. V-grade filters are used mainly for 'clarifying' liquids but they are not fine enough to sterilise liquids by holding back bacteria. American Mandler filters are made from kieselguhr, asbestos and plaster of paris. These are similar to the Berkefeld filters.

These filters are usually made in the form of hollow candles open at one end. A metal nozzle is cemented in the open end. The liquid to be filtered is either poured into the candle or passed through the candle from without.

Porcelain filters: The most common type of porcelain filter is the Chamberland, a French filter made of kaolin and sand, and the English, Doulton. The Chamberland filters are made in various porosities which are graded L1, L1a, L2, L3, L5, L7, L9 and L11. L1 is clarifying and equal to Berkefeld V. The others are for sterilising. The Doulton filter candle range is P2, P5 and P11, the latter two being sterilising filters.

For use, the candle is mounted on a silicone rubber bung, and filtration is effected from within outwards.

Asbestos filters (Seitz): This is made from the chrysotile type of asbestos. Chemically, this is mainly composed of magnesium silicate. The filter disc is supported on a metal mount and is inserted rough side up ensuring that the metal support grid is in position. The filter is attached to a vacuum flask through a silicone rubber bung.

The side arm of the flask is plugged with non-absorbent cotton wool and the filter unit is wrapped in Kraft paper and autoclaved. After use, the disc is discarded. Each time a fresh disc is used and the outfit sterilised by autoclaving.

The asbestos discs are available in different grades. The more important ones are:

1. HP/PYR : For removal of pyrogens
2. HP/EKS : For absolute sterility using controlled standard conditions
3. HP/EK : For clarifying

Sintered glass filters: These are made of finely ground glass which is fused to make the particles adhere. These are available in different pore sizes as per British Standards specification.

Membrane filters: Two types of these are available. The older type, gradocol membranes, (graded collodion membrane of Elford) are composed of cellulose nitrate whereas the modern membrane filters consist of cellulose acetate.

The gradocol membranes are made from an acetone solution of collodion diluted with ethyl alcohol ether mixture to which are added varying amounts of amyl alcohol. The mixture is poured into a shallow cell in a room with constant temperature (22.5°C) and allowed to evaporate for varying periods of 1–3 hours and then washed over an extended period with distilled water. By varying the amount and composition of collodion mixture and the conditions of evaporation, filters of average pore size (APS) ranging from 3μ to 10mμ or less have been prepared. These filters are sterilised by autoclaving at 121°C for 15 minutes.

Radiation

Two types of radiation are used for sterilising purposes: nonionising and ionising. Infrared and ultraviolet rays are of the nonionising low energy type while gamma rays and high energy electrons are of the high energy ionising type.

Nonionising radiation: In nonionising radiation electromagnetic rays with wavelengths longer than those of visible light are used and these are to a large extent absorbed as heat. Hence infrared radiation can be considered as a form of hot air sterilisation. Infrared radiation is used for rapid mass sterilisation of syringes. Ultraviolet radiation is used for disinfecting enclosed areas

such as entryways, hospital wards, operation rooms and small virus inoculation rooms and virus laboratories.

Ionising radiation: X-rays, gamma rays and cosmic rays are highly lethal to DNA and other vital cell constituents. They have very high penetrative power. Since there is no appreciable increase in temperature, this method is referred to as *cold sterilisation*. Large commercial plants use gamma radiation for sterilising most plastics, syringes, swabs, culture plates, catheters, animal feeds, various types of rubber, cardboard, oils, greases, fabrics and metal foils.

High energy electron radiation as a method of sterilisation is not widely used in medicine.

Ultrasonic and sonic vibration

Ultrasonic and sonic waves are credited with bactericidal powers but the results are variable. Microorganisms vary in their sensitivity to them and survivors are found after such treatment. Hence this method is of no practical value in sterilisation and disinfection.

Chemical agents

We have a bewildering number and variety of chemical agents used as antiseptics and disinfectants. Remarkably little is known about the mechanism of action of many of these agents.

An ideal antiseptic or disinfectant should

1. have a wide spectrum of activity and be effective against all microorganisms, viz., bacteria including spores, viruses, protozoa and fungi;
2. be active in the presence of organic matter;
3. be effective in acid as well as alkaline media;
4. have speedy action;
5. have high penetrating power;
6. be stable;
7. be compatible with other antiseptics and disinfectants;
8. not corrode metals;
9. not cause *local irritation or sensitisation*;
10. not interfere with healing;
11. not be toxic if absorbed into circulation;
12. be cheap and easily available; and
13. be safe and easy to use.

Such an ideal chemical has yet to be found.

The factors that determine the potency of disinfectants are:

1. concentration of the substance,
2. time of action,
3. pH of the medium,
4. temperature,
5. nature of the organisms, and
6. presence of extraneous material.

These substances act in various ways. The main modes of action are:

1. protein coagulation;
2. disruption of cell membrane, thus resulting in exposure of the contents of the cell to the adverse environment and loss of the constituents of and changes in the composition of the contained cytoplasm. These cause the death of the cell;
3. removal of free sulphydryl groups which are essential for the functioning of the enzymes and thus the life of the cell; and
4. substrate competition. A compound resembling the essential substrate of the enzyme diverts the enzymes or misleads the enzymes necessary for the metabolism of the cell and causes cell death.

Alcohols

Ethyl alcohol (ethanol) and isopropyl alcohol are the most frequently used. They are used mainly as skin antiseptics and act by denaturing bacterial proteins. They have no action on spores or viruses. To be effective, they must be used at a concentration of 60–70 per cent in water. Protein slows its action whereas 1% mineral acid or alkali enhances the activity. Isopropyl alcohol is preferred to ethyl alcohol as it is a better fat solvent, more bactericidal and less volatile. It is used for the disinfection of clinical thermometers.

Methyl alcohol is effective against fungal spores and is used for treating cabinets and incubators affected by them. The insides of the

chambers are liberally wiped with methanol and a pad moistened with it, along with a dish of water (to ensure high humidity) kept inside, and the incubator is left at working temperature for several hours. One must remember that methyl alcohol vapour is toxic and inflammable.

Aldehydes

Formaldehyde (HCHO) is active against the amino group in the protein molecule. In aqueous solutions, it is markedly bactericidal and sporicidal and also has a lethal effect on viruses. It is used to preserve anatomical specimens, and for destroying anthrax spores in hair and wool; 10% formalin containing half per cent sodium tetraborate is used to sterilise clean metal instruments.

Formaldehyde gas is used for sterilising instruments and heat sensitive catheters. It is used for fumigating wards, sick rooms and laboratories. Under properly controlled conditions, clothing, bedding, furniture and books can be satisfactorily disinfected.

The gas is irritant and toxic when inhaled. Surfaces which have been disinfected by this agent may give an irritant vapour for some time after disinfection, and this can be nullified by exposure to ammonia vapour when disinfection has been completed.

Glutaraldehyde (CHO–$(CH_2)_3$–CHO): This has an action similar to formaldehyde. It is specially effective against tubercle bacilli, fungi and viruses. It is less toxic and irritant to the eyes and skin than formaldehyde. This has no deleterious effect on the cement or lenses of instruments such as cystoscopes and bronchoscopes. It can be safely used to treat corrugated rubber anaesthetic tubes and face masks, plastic endotracheal tubes, metal instruments and polythene tubing.

Dyes

Two groups of dyes, a) the aniline dyes, and b) the acridines are used extensively as skin and wound antiseptics. Both are bacteriostatic in high dilution but are of low bactericidal activity. The aniline dyes in use are brilliant green, malachite green and crystal violet. They are more active against Gram positive than Gram negative organisms. They have no activity against tubercle bacilli, and hence the use of malachite green in Lowenstein-Jensen medium. Though they are nonirritant to the tissues and nontoxic, they are considerably inhibited by organic material such as pus. Their lethal effects on bacteria are believed to be due to their reaction with the acid groups in the cell. These dyes are used in the microbiology laboratory as selective agents in culture media.

The acridine dyes are more active against Gram positive than Gram negative organisms but not as selective as aniline dyes. They are very little affected by the presence of organic matter. The more important dyes are proflavine, acriflavine, euflavine and aminacrine. They show no significant differences in potency. If impregnated in gauze they are slowly released in a moist environment, and hence their advantage and use in clinical medicine. They impair the DNA complexes of the organisms and thus kill or destroy the reproductive capacity of the cell.

Halogens

Iodine in aqueous and alcoholic solutions has been used as a skin disinfectant. It is an active bactericidal agent with a moderate activity against spores. It is active against the tubercle bacillus and a number of viruses. Compounds of iodine with nonionic wetting or surface active agents known as iodophores, are claimed to be more active than the aqueous or alcoholic solutions of iodine.

Chlorine and its compounds have been used as disinfectants for many years. Water supplies, swimming baths, food and dairy industries are some of the areas of their use. Chlorine is used most commonly as hypochlorites. Chlorine and hypochlorites are markedly bactericidal. They have a wide spectrum of activity against viruses.

The organic chloramines are used as antiseptics for dressing wounds.

Phenols

These are obtained by distillation of coal tar between temperatures of 170°C and 270°C. Lister, the father of antiseptic surgery, first introduced their use in surgery (1865). Since then a wide range of phenolic compounds have been developed as disinfectants. The lethal effect of phenols is due to their capacity to cause cell membrane damage, thus releasing cell contents and causing lysis. Low concentrations of phenol precipitate proteins, and membrane-bound oxidases and dehydrogenases are irreversibly inactivated by concentrations of phenol that are rapidly bactericidal for the organism.

Phenol (carbolic acid) is a powerful microbicidal substance. This and other phenolic disinfectants derived from coal tar are widely used as disinfectants for various purposes in hospitals. Lysol and cresols are active against a wide range of organisms. They are not readily inactivated by the presence of organic matter and are thus good general disinfectants. These are markedly toxic to man. Various proprietary preparations or formulations of phenol are in wide use. The related chlorophenols and chloroxyphenols, though less toxic and irritant, are less active and more readily inactivated by organic matter. Both these groups of substances are relatively inactive against Pseudomonas. Various combinations of this are used in the control of pyogenic cocci in surgical and neonatal units in hospitals. Hexachlorophene is potentially toxic and should be used with care. Chlorhexidine (Hibitane) is a relatively nontoxic skin antiseptic most active against Gram positive organisms and fairly effective against Gram negative ones. Aqueous solutions are used in the treatment of wounds.

Gases

Ethylene oxide: This is a colourless liquid with a boiling point of 10.7°C, and at normal temperature and pressure is a very penetrating gas with a sweet ethereal smell. It is highly inflammable and in concentrations in air greater than three per cent, highly explosive. By mixing it with inert gases such as carbon dioxide or nitrogen, so that its concentration is only 10 per cent, its explosive tendency is eliminated.

Its action is due to its power of alkylating the amino, carboxyl, hydroxyl and sulphydryl groups in the protein molecule. In addition, it reacts with DNA and RNA. Its use as a disinfectant presents some potential hazard of toxicity to man including mutagenicity and carcinogenicity. It is effective against all types of microorganisms including viruses and spores.

It diffuses through many types of porous materials and readily penetrates some plastics. It is specially used for sterilising heart-lung machines, respirators, sutures, dental equipment, books and clothing. It is unsuitable for fumigating rooms because of its explosive property. It has been successfully used to sterilise a wide range of materials such as glass, metal and paper surfaces, clothing, plastics, soil, some foodstuffs and tobacco. It is an irritant, and personnel working with it have to take strict precautions.

Formaldehyde gas: This is widely employed for fumigation of operation theatres and other rooms. After sealing windows and other outlets, formaldehyde gas is generated by adding 150 grams $KMnO_4$ to 280 ml formalin for every 1000 cu.ft (28.3 cu. metre) of room volume. The reaction produces considerable heat, and so heat resistant vessels should be used. After starting generation of formaldehyde vapour, the doors should be sealed and left unopened for 48 hours.

Betapropiolactone (BPL): This a condensation product of ketane and formaldehyde with a boiling point of 163°C. Though as a gas it has a low penetrating power, it is said to be more efficient for fumigating purposes than formaldehyde. It has a rapid biocidal action but unfortunately has carcinogenic activity. 0.2 per cent BPL is used for the sterilisation of biological products. It is capa-

ble of killing all microorganisms and is very active against viruses.

Surface active agents

Substances which alter energy relationships at interfaces, producing a reduction of surface or interfacial tension are referred to as surface active agents. They are widely used as wetting agents, detergents and emulsifiers. They are classified into four main groups, viz., anionic, cationic, nonionic and amphoteric. The most important antibacterial agents are the cationic surface active agents. These act on the phosphate groups of the cell membrane and also enter the cell. The membrane loses its semipermeability and the cell proteins are denatured. The cationic compounds in the form of quaternary ammonium compounds are markedly bactericidal, being active against Gram positive organisms and to a less extent on Gram negative ones. They have no action on spores, tubercle bacilli and most viruses. The common compounds are cetyl trimethyl ammonium bromide (Cetavlon or cetrimide) and benzalkonium chloride. These compounds are most active at alkaline pH. Acid inactivates them Organic matter reduces their activity and anionic surface active agents, like ordinary soaps, render them inactive. The anionic compounds, e.g., common soap, have a moderate activity. Soaps prepared from saturated fatty acids (e.g., coconut oil) are more effective against Gram negative bacilli while those prepared from unsaturated fatty acids (e.g., oleic acid) have greter activity against Gram positive and Neisseria group of organisms. The amphoteric or ampholytic compounds, known as 'Tego' compounds, are active against a wide range of Gram positive and Gram negative organisms and some viruses. These, however, are not in general use.

Metallic salts

Though all salts have a certain amount of germicidal action depending on their concentration, salts of heavy metals have a greater action: The salts of silver, copper and mercury are used as disinfectants. They are protein coagulants and have the capacity to combine with free sulphydryl groups of cell enzymes, when used at appropriate concentrations. Mercuric chloride is used in medicine but is highly toxic. The organic compounds, thiomersal, phenyl mercury nitrate and mercurochrome are less toxic and are used as mild antiseptics and have a marked bacteriostatic, limited fungicidal and a weak bactericidal activity. Silver salts in aqueous solution have a limited use. Copper salts are used as fungicides.

Testing of disinfectants

There is no single test available to determine the efficiency of a disinfectant. This is due to the number of parameters which have to be taken into account in determining the quality of the disinfectant. In the Rideal Walker test, similar quanitities of organisms are submitted to the action of varying concentrations of phenol and of the germicide to be tested. The dilution of the disinfectant in question which sterilises the suspension in a given time is divided by the dilution of phenol which sterilises the suspension at the same time and the phenol coefficient is obtained (Phenol = 1). This test has many limitations as the conditions under which the test is done have no relation to the conditions under which the disinfectant in question has to act. In the Chick Martin test, unlike in the former where the disinfectant acts in pure solution, the disinfectant is tested in water containing a suspension of 3% dried human faeces. Even this modification falls far short in simulating the conditions in which disinfectants are called upon to act. Various other modifications are in use, but no test is comprehensive enough.

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4 Culture Media

It is essential to grow the organisms from infected material to identify the cause of infection Only after growing them and isolating them in pure culture, is it usually possible to identify them. For studying their characteristics as well, it is necessary to culture them. It is not always possible to identify organisms by morphology alone.

The majority of bacteria to be studied are pathogenic. Hence to obtain suitable growth of the bacteria, the culture media should approximate to the composition and reaction of the tissues and body fluids in which these bacteria grow. No single medium can satisfy all the requirements. A medium could be perfect only for a limited range. The food requirements of bacteria vary with their natural environments and the particular role they play in nature. They derive their energy by oxidation and by decomposition of food material such as proteins and carbohydrates. At the same time simple organic substances like aminoacids are utilised in the synthetic metabolism of these organisms.

Bacteria vary considerably in their nutritional requirements, and over seven thousand culture media have been devised. The basic requirements of culture media are: 1) energy source, 2) carbon source, (For the great majority of the organisms the same material serves both these purposes There is, however, great variation as to the source from which carbon can be utilised.) 3) nitrogen source, 4) salts such as sulphates, phosphates, chlorides, carbonates of K, Na, Mg, Fe and Ca and trace elements such as Mn, Mo, Cu, 5) a satisfactory pH, usually 7.2–7.6, adequate oxidation-reduction potential (Eh), 7) growth factors, e.g., Tryptophan for *S. typhi*, Glutathione for gonococci, X and V factors for Haemophilus.

The characteristics of an ideal culture medium are that it

1. must give a satisfactory growth from a small inoculum and ideally from a single cell;
2. should give a rapid growth;
3. should be easy to prepare;
4. should be reasonably cheap;
5. should be easily reproducible;
6. should make it possible for all the characteristics in which we are interested to be demonstrated.

It was Louis Pasteur who first introduced the use of complex media. Urine, chicken broth and meat broth were in use. All were liquid media and though growth could be obtained, growth characteristics and purity of cultures could not be made out. Robert Koch introduced solid media. He used cut potato. Then he used gelatin to get solid media. Since gelatin melts at 24°C and often gets liquefied by the growing organisms it was found unsatisfactory. On the suggestion of Frau Hesse, he introduced agar agar as a base for preparing solid media. Agar is prepared from varieties of seaweed. The chief constituent is a long-chain polysaccharide. It also contains varying amounts of inorganic salts and protein-like materials. Agar gets hydrolysed at a high temperature at acid or alkaline pH. It melts at 98°C and usually sets at 42°C depending on agar concentration. At lower concentrations, e.g., sloppy agar, it sets only at 35°C. New Zealand agar has twice the jellifying capacity of Japanese agar. Agar is manu-

factured either in long shreds or in powder form. It varies in its composition from batch to batch and also depending on its source.

Another almost universal ingredient of common media is peptone. It is a complex mixture of partially digested proteins. Its constituents are proteoses, polypeptides and aminoacids, a variety of inorganic salts including phosphates, potassium and magnesium and certain accessory growth factors such as riboflavin. Different brands of peptone show appreciable differences in composition and growth-promoting properties. There may be variations between different batches of the same brand. Special brands of peptone such as Neopeptone, proteose peptone are available for special uses. Commercially available peptones or digest broth can be used. Meat extract is also available commercially and is known as Lab-Lemco. Blood, serum and yeast extract are other common ingredients.

Types of media

Media have been classified in many ways:

1. Solid media, liquid media, semi-solid media.
2. Simple media, complex media, synthetic or defined media, semi-defined media, special media. Special media are further divisible into:

   1) enriched media, 2) enrichment media, 3) selective media, 4) indicator or differential media, 5) sugar media, and 6) transport media.
3. Aerobic media, anaerobic media.

Simple media (Basal media): An example is nutrient broth. It consists of peptone, meat extract, sodium chloride and water. Nutrient agar, made by adding 2% agar to nutrient broth is the simplest and commonest medium in routine diagnostic laboratories. If the concentration of agar is reduced to 0.2–0.5 per cent, semisolid or sloppy agar is obtained which enables motile organisms to spread. Increasing the concentration of agar to 6% prevents spreading or swarming by organisms such as Proteus.

Complex media: These have added ingredients for special purposes or for bringing out certain characteristics or providing special nutrients required for the growth of the bacterium in question. One may say that all media other than the simple are complex. All special media can come under this heading.

Synthetic or defined media: These media are prepared solely from pure chemical substances and the exact composition of the medium is known. These are used for various special studies such as metabolic requirements. Simple peptone water medium, 1% peptone with 0.5% NaCl in water, may be considered as a semi-defined medium since its composition is approximately known.

Enriched media: In these media, substances such as blood, serum, egg are added to a basal medium. These media are used to grow bacteria which are more exacting in their nutritional needs. Examples are blood agar, chocolate agar, and egg media.

Enrichment media: In mixed cultures or in materials containing more than one bacterium, the bacterium to be isolated is often overgrown by the unwanted bacteria. Usually the non-pathogenic or commensal bacteria tend to overgrow the pathogenic ones, e.g., *S. typhi* being over grown by *E. coli* in cultures from faeces. In such situations substances are incorporated in the medium which have a stimulating effect on the bacteria to be grown or an inhibitory effect on those to be suppressed. If such substances are added to a liquid medium, the result is an absolute increase in the numbers of the wanted bacterium relative to the other bacteria. Such media are called enrichment media, e.g., tetrathionate broth where the tetrathionate inhibits coliforms while allowing typhoid-paratyphoid bacilli to grow freely. So also is Selenite F broth.

Selective media: If in the above case the inhibiting substance is added to a solid medium, it enables a

greater number of the required bacterium to form colonies than the other bacteria, e.g., desoxycholate citrate medium for dysentery bacilli. Such solid media are known as selective media.

Indicator media: These media contain an indicator which changes colour when a bacterium grows in them, e.g., incorporation of sulphite in Wilson and Blair medium. *S. typhi* reduces sulphite to sulphide in the presence of glucose and the colonies of *S. typhi* have a black metallic sheen. Potassium tellurite in McLeod's medium gets reduced to metallic tellurium by diphtheria bacillus to produce black colonies.

Differential media: A medium which has substances incorporated in it enabling it to bring out differing characteristics of bacteria and thus helping to distinguish between them is called 'differential medium', e.g., MacConkey's medium which consists of peptone, lactose, agar, neutral red and taurocholate shows up lactose fermenters as pink colonies while nonlactose fermenters are colourless or pale. This may also be termed as indicator medium

Some of these terms for media are interchangeable. For example, the blood agar medium is an enriched medium but bacteria lysing red cells give clearing around their colonies. Thus, it is an indicator medium as well. There are many special media for demonstrating particular characteristics, like Nagler's medium which enables lecithinase activity to be brought out.

Sugar media: The term sugar in microbiology denotes any fermentable substance. They may be:

1. Monosaccharides — (a) Pentoses, e.g., Arabinose, Xylose, (b) Hexoses, e.g., Dextrose, Mannose
2. Disaccharides, e.g., Saccharose, Lactose
3. Polysaccharides, e.g., Starch, Inulin
4. Trisaccharides, e.g., Raffinose
5. Alcohols, e.g., Glycerol, Sorbitol
6. Glucosides, e.g., Salicin, Aesculin
7. Noncarbohydrate substance, e.g., Inositol

The usual sugar media consist of 1% of the sugar concerned in peptone water along with an appropriate indicator. A small tube (Durham's tube) is kept inverted in the sugar tube to detect gas production. For organisms which are exacting in their growth requirements (e.g., pneumococci) Hiss' serum sugars are used. They contain 3% serum.

Transport media: In the case of delicate organisms (e.g., gonococci) which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by nonpathogens (e.g., dysentery or cholera organisms), special media are devised and these are termed transport media, e.g., Stuart's medium — a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralise certain bacterial inhibitors — for gonococci; buffered glycerol saline for enteric bacilli.

Anaerobic media: These media are used to grow anaerobic organisms, e.g., Robertson's cooked meat medium.

Various media to test special properties like urease production, and miscellaneous tests for special properties have been devised. They are dealt with in the appropriate chapters.

For identifying prepared media, a colour code is adopted. This depends on the laboratory or group of laboratories. One colour or a mixture of colours is used on the cotton stopper, or colour paints are used on the screw caps. Different colours or colour combinations are used for different media.

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5 Culture Methods

Culture methods employed depend on the purpose for which they are intended. In the clinical laboratory, the indications for culture are mainly to

1. isolate bacteria in pure culture
2. demonstrate their properties,
3. obtain sufficient growth for preparation of antigens and for other tests,
4. type isolates by methods such as bacteriophage and bacteriocin susceptibility,
5. determine sensitivity to antibiotics,
6. estimate viable counts, and
7. maintain stock cultures

The methods of culture used ordinarily in the laboratory are the streak, lawn, stroke, stab, pour plate and liquid cultures. Special methods are employed for culturing anaerobic bacteria. For estimating the bacteria in dust on clothing, the *sweep plate* method is used.

The *streak culture* (surface plating) is the method routinely employed for the isolation of bacteria in pure culture from clinical specimens. A platinum loop is charged with the specimen to be cultured. Owing to the high cost of platinum, loops for routine work are made of Nichrome resistance wire (24 S.W.G. size). One loopful of the specimen is transferred onto the surface of a well dried plate, on which it is spread over a small area at the periphery. The inoculum is then distributed thinly over the plate by streaking it with the loop in a series of parallel lines, in different segments of the plate. The loop should be flamed and cooled between the different sets of streaks. On incubation, growth may be confluent at the site of original inoculation, but becomes progressively thinner, and well separated colonies are obtained over the final series of streaks.

The *lawn* or *carpet culture* provides a uniform surface growth of the bacterium and is useful for bacteriophage typing and antibiotic sensitivity testing (disc method). It may also be employed when a large amount of growth is required in solid media as, for instance, in the preparation of bacterial antigens and vaccines. Lawn cultures are prepared by flooding the surface of the plate with a liquid culture or suspension of the bacterium, pipetting off the excess inoculum and incubating the plate. Alternatively, the surface of the plate may be inoculated by applying a swab soaked in the bacterial culture or suspension.

The *stroke culture* is made in tubes containing agar slope (slant) and is employed for providing a pure growth of the bacterium for slide agglutination and other diagnostic tests.

Stab cultures are prepared by puncturing with a long, straight charged wire, a suitable medium such as nutrient gelatin or glucose agar. The medium is allowed to set, with the tube in the upright position, providing a flat surface at the top of the medium. Stab cultures are employed mainly for demonstration of gelatin liquefaction and oxygen requirement of the bacterium under study. They are also used in the maintenance of stock cultures.

For preparing *pour plate culture*, tubes containing 15 ml of the agar medium are melted and left to cool in a water bath at 45–50°C. Appropriate dilutions of the inoculum are added in 1 ml volume, to the molten agar, mixed well and the contents of the tube poured into a sterile Pet-

ri dish and allowed to set. After incubation, colonies will be seen well distributed throughout the depth of the medium and can be enumerated using colony counters. The pour plate method gives an estimate of the viable bacterial count in a suspension and is the recommended method for quantitative urine cultures.

In the *sweep plate method*, the edges of the Petri dishes containing the culture medium are rubbed over the fabric with the medium facing it. The dust particles stirred up from the cloth get settled on the culture medium, and colonies develop on incubation. They can be counted and estimates made.

Liquid cultures in tubes, bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculum with pipettes or syringes. Large inocula can be employed in liquid cultures and hence this is the method adopted for blood culture and for sterility tests, where the concentration of bacteria in the inocula are expected to be small. Liquid cultures are preferable for inocula containing antibiotics and other antibacterial substances, as these are rendered ineffective by dilution in the medium. Liquid cultures are also preferred when large yields are desired, the yield being enhanced by agitation, aeration, addition of nutrients and removal of toxic metabolites (continuous culture methods). The major disadvantage of liquid culture is that it does not provide a pure culture from mixed inocula.

Anaerobic culture methods

Anaerobic bacteria differ in their requirement of and sensitivity to oxygen. Some, such as *Cl. histolyticum*, are aerotolerant and may produce some growth on the surface of aerobic plates, while others, such as *Cl. tetani*, are strict anaerobes and form surface growth only if the oxygen tension is less than 2 mm Hg. A number of methods have been described for achieving anaerobiosis, the principles being exclusion of oxygen or production of a vacuum, displacement of oxygen with other gases, absorption of oxygen by chemical or biological means, and reduction of oxygen.

1. Cultivation in vacuum was attempted by incubating cultures in a vacuum desiccator, but the method is unsatisfactory as some oxygen always remains behind. Fluid cultures may boil over and the media may get detached from the plates in the vacuum produced. This method is not in use now.

2. Displacement of oxygen with gases such as hydrogen, nitrogen, helium or carbon dioxide is sometimes employed, but anaerobiosis is seldom complete by this method alone. A popular, but ineffective, method is the candle jar. Here inoculated plates are placed inside a large air-tight container and a lighted candle kept in it before the lid is sealed. The burning candle is expected to use up all the oxygen inside before it is extinguished, but some oxygen is always left behind. The candle jar provides a concentration of carbon dioxide which stimulates the growth of most bacteria.

3. In the chemical or biological method, alkaline pyrogallol absorbs oxygen. This method, first introduced by Buchner (1888), has been employed with different modifications for providing anaerobiosis. Pyrogallic acid added to a solution of sodium hydroxide in a large test tube placed inside an air-tight jar provides anaerobiosis, but a small amount of carbon monoxide, which is formed during the reaction, may be inhibitory to some bacteria.

The method has been applied to single tube and plate cultures. The Spray anaerobic dish is a glass dish with its bottom partitioned into two halves, and the top accommodating one half of a Petri dish carrying the medium. Pyrogallic acid and sodium hydroxide are placed in the separate halves at the bottom of the dish. The inoculated culture plate is inverted on the top of the dish and sealed air-tight with plasticine. The dish is then rocked to mix the reagents, producing anaerobiosis. The anaerobic dish is not in use now.

A simple modification consists of a Petri dish, between the two halves of which is inserted a metal disc of slightly larger diameter, with a hole

in the centre. The metal disc is attached to the bottom half of the Petri dish with plasticine. Through the central hole are added a few pellets of sodium hydroxide and 10 ml of a 10% solution of pyrogallic acid. The inoculated half of the Petri dish is then inverted on the metal disc and sealed *air-tight.*

The method in common use employs a disc of filter paper having the same diameter as a Petri dish. It is placed on top of one half of the dish and a mixture of pyrogallol and sodium carbonate, in dry powder form, is spread on it. The inoculated plate is inverted over the filter paper and sealed tight with molten wax. The dry pyrogallol mixture is activated by the moisture within the closed system, and complete anaerobiosis develops within about two hours.

Instead of alkaline pyrogallol, anaerobiosis has been produced within jars with a mixture of chromium and sulphuric acid (Rosenthal method) or with yellow phosphorous.

Absorption of oxygen from small closed systems has been attempted by incubation along with aerobic bacteria, germinating seeds or chopped vegetables. Anaerobiosis produced by such biological methods is slow and ineffective.

The most reliable and widely used anaerobic method is the McIntosh and Fildes' anaerobic jar (Fig. 5.1.). It consists of a stout glass or metal jar with a metal lid which can be clamped air-tight with a screw. The lid has two tubes with taps, one acting as the gas inlet and the other as the outlet. The lid also has two terminals which can be connected to an electrical supply. Leading from the terminals and suspended by stout wires on the underside of the lid is a small grooved porcelain spool around which is wrapped a layer of palladinised asbestos.

Inoculated culture plates are placed inside the jar, with the medium in the bottom half of the plates, and the lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the inlet tube connected to a hydrogen supply. After the jar is filled with hydrogen, the electric terminals are connected to a current supply so that the

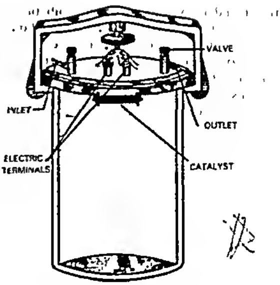

Fig. 5.1 McIntosh-Fildes' Jar

palladinised asbestos is heated. This acts as a catalyst for the combination of hydrogen with the residual oxygen present in the jar. This method ensures complete anaerobiosis, but carries the risk of explosion, which may rarely occur. This risk can be eliminated by modification of the catalyst. Alumina pellets coated with palladium and contained in a gauze sachet suspended from the lid of the jar act as a catalyst at room temperature, as long as the sachet is maintained dry.

The 'Gaspak' is now the method of choice for preparing anaerobic jars. The 'Gaspak' is commercially available as a disposable envelope, containing chemicals which generate hydrogen and carbon dioxide on the addition of water. After the inoculated plates are kept in the jar, the 'Gaspak' envelope, with water added, is placed inside and the lid screwed tight. Hydrogen and carbon dioxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment. The 'Gaspak' is simple and effective, eliminating the need for drawing a vacuum and adding hydrogen.

An indicator should be employed for verifying the anaerobic condition in the jars. Reduced methylene blue is generally used for this purpose.

It remains colourless anaerobically, but turns blue on exposure to oxygen.

4. Reduction of oxygen in the medium is achieved by the use of various reducing agents, including 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid and 0.05% cysteine. An easily prepared anaerobic medium is broth into which is introduced pieces of red hot metallic iron, the broth being then layered over with sterile vaseline. Broth containing fresh animal tissue, such as rabbit kidney, spleen, testes or heart (Smith-Noguchi medium) supports the growth of many anaerobes.

Robertson's cooked meat medium is probably the most widely used fluid medium for the culture of anaerobes. It consists of fat-free minced cooked meat in broth, with a layer of sterile vaseline over it. It permits the growth of even strict anaerobes and indicates their saccharolytic or proteolytic activities, by the meat being turned red or black, respectively.

For fastidious anaerobes, particularly for quantitative cultures, pre-reduced media and anaerobic chamber ('glove box') may be used. The anaerobic chamber is an airtight, glass-fronted cabinet filled with inert gas, with an entry lock for the introduction and removal of materials, and gloves for the insertion of hands for working.

Methods of isolating pure cultures

The following methods may be employed for isolating pure cultures of bacteria from mixtures:

1. Surface plating is the method routinely employed in clinical bacteriology and enables the isolation of distinct colonies which may be picked out, if necessary, for further purification and study.

2. Enrichment, selective and indicator media are widely used for the isolation of pathogens from specimens such as faeces, with varied flora.

3. Pure cultures may be obtained by pre-treatment of specimens with appropriate bactericidal substances which destroy the unwanted bacteria. This method is the standard practice for isolation of tubercle bacilli from sputum and other clinical specimens, by treatment with alkali, acid or other substances to which most commensals are susceptible but tubercle bacilli are resistant.

4. Obligate aerobes and anaerobes may be separated by cultivation under aerobic or anaerobic conditions. Shake cultures in Veillon tubes were in use formerly but are now obsolete. This consists of a glass tube open at both ends. One end is closed with a rubber stopper and molten glucose agar in which the inoculum is evenly dispersed is poured into the tube and allowed to set in a vertical position. The top of the tube is closed with a cotton plug. On incubation, the bacteria in the inoculum differentiate depending on their oxygen requirement. The obligate aerobes grow at the top and the anaerobes at the bottom, while the facultative bacteria grow throughout the column. The entire medium can be extruded on to a plate and the different colonies fished out.

5. Separation of bacteria with different temperature optima can be effected by incubation at different temperatures. Only thermophilic bacteria grow at 60°C. A mixture containing *Neisseria meningitidis* and *N. catarrhalis* can be purified by incubation at 22°C when only the latter grows.

6. By heating at 80°C a mixture containing vegetative and spore-forming bacteria, the former can be eliminated. This method is useful for the isolation of tetanus bacilli from dust and similar sources.

7. Separation between motile and nonmotile bacteria can be effected using the Craigie's tube. This consists of a tube of semi-solid agar, with a narrow tube open at both ends placed in the centre of the medium in such a way that it projects above the level of the medium. The mixture is inoculated into the central tube. On incubation, the motile bacteria alone traverse the agar and appear on the top of the medium outside the central tube. A U-tube also serves the same purpose, inoculation being performed into one limb and the subculture taken from the other. This method can also be used to obtain phase variants in Salmonella species.

8. Pathogenic bacteria may be isolated from

mixtures by inoculation into appropriate animals. Anthrax bacilli can be distinguished from other aerobic sporulating bacilli by inoculation into mice or guinea pigs. Anthrax bacilli produce a fatal septicaemia and may be cultured pure from the heart blood.

9. Bacteria of differing sizes may be separated by the use of selective filters. Filters are widely used for separating viruses from bacteria.

10. Single-cell method using micromanipulators, by which a single bacterium can be separated and cultured, are too complicated for routine use, but may find application in special situations.

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6 Identification of Bacteria

Once a bacterium has been obtained in pure culture, it has to be identified. This may be easy but is often difficult and sometimes may take quite a few days. The following characteristics have to be studied in the process.

Morphology

The morphology of the organism depends on a number of factors such as the strain studied, nature of the culture medium, temperature and time of incubation, age of the culture and the number of subcultres it has undergone. The characteristics noted are shape, size, arrangement, motility, flagella, spores and capsules. All these cannot be made out in a single medium. The shape may be spherical, rod-like, filamentous, comma-like or spiral. The axis of the organism may be straight or curved. The length and breadth may vary. The sides of the organism may be parallel; convex, concave, or irregular. The ends may be cut straight, rounded or tapering. *Considerable variations in shape and size* leading to club-like forms, navicular forms, and swollen or shadow or giant forms may be seen. They may be arranged singly, in pairs, in tetrads or in packets of eight or in chains, short or long, in the case of cocci; bacilli may be arranged irregularly, in short or long chains, in Chinese letter patterns, palisade-like or in bundles; vibrios may be single or in S or spiral forms. They may be nonmotile, sluggishly motile, actively motile or exhibit darting motility. They may be without flagella, i.e., atrichate, or monotrichate, lophotrichate, amphitrichate or peritrichate. The spores, when present, may be oval or *spherical* or ellipsoidal and may be of the same width or wider than that of the bacillus. The spores may be equatorial, subterminal or terminal. Capsules may or may not be present. Hanging drop preparations, dark ground illumination, phase contrast or electron microscopy, all help in these studies.

Staining reactions

The age of the culture is important. In older cultures, staining characteristics either vary or are not brought out well. Simple stains bring out the morphology best. Differential and special stains are necessary to bring out characteristics like flagella, capsules, spores and metachromatic granules. The Gram stain divides bacteria into Gram positive and Gram negative; the Ziehl-Neelsen stain into acid fast and nonacid fast. Fluorescent antibody technique enables one to identify them.

The study of morphology and staining characteristics helps in preliminary identification.

Cultural characteristics

These provide additional information for the identification of the bacterium. The characters revealed in different types of media are noted. While studying colonies on solid media,

1. their shape — whether circular, irregular, radiate or rhizoid,
2. size in millimeters,
3. their elevation — whether effuse, elevated, low convex, concave, umbonate or umbilicate,

4. their margins — bevelled or otherwise,
5. their surface — smooth, wavy, rough, granular, papillate or glistening,
6. their edges — entire, undulate, crenated, fimbriate or curled,
7. their colour,
8. their structure — whether opaque, translucent or transparent,
9. their consistency — whether membranous, friable, butyrous or viscid,
10. their emulsifiability, and
11. whether they are differentiated into a central and a peripheral portion

are noted.

In a stroke culture,

1. the degree of growth — scanty, moderate, or profuse,
2. their nature — either discrete or confluent, filiform, spreading or rhizoid,
3. their elevation, surface, edges, colour, structure, odour, emulsifiability, consistency and changes in the medium

are noted.

In a fluid medium, the degree of growth — absence, scanty, moderate or abundant — presence of turbidity and its nature, presence of deposit and its character, nature of surface growth such as pellicle and its quality and ease of disintegration, and odour are noted.

Resistance

The resistance of the organism to heat and to low concentrations of disinfectants is tested, both for vegetative and spore forms. Resistance of *Str. faecalis* to heat at 60°C for half an hour and of clostridial spores to boiling for various periods are examples. Resistance to antibiotic and chemotherapeutic agents and bacteriocins would also help in differentiation and identification.

Metabolism

The requirements of oxygen, the need for CO_2, capacity to form pigments, and powers of haemolysis help us to classify bacteria and differentiate species.

Fermentation and other biochemical properties

The more important and widely used tests are described below:

1. Sugar fermentation: This is tested in sugar media. Acid production is shown by change in the colour of the medium to pink or red, and the gas produced collects in Durham's tube.

2. Litmus milk: There may be no change in the medium, or acid or alkali may be produced; clotting of milk, peptonisation or saponification may occur. The clot may be disrupted by gas produced (stormy fermentation).

3. Indole production: This is tested in a peptone water culture after 48 or 96 hours incubation at 37°C. This test demonstrates the production of indole from tryptophane. Add 0.5 ml Kovac's reagent and shake gently. A red colour indicates a positive reaction. Kovac's reagent consists of:

Paradimethylaminobenzaldehyde	10 g
Amyl or isoamyl alcohol	150 ml
Conc. HCl	50 ml

This is prepared in small quantities and stored in the refrigerator.

4. Methyl red test (MR): This test is employed to detect the productinn of acid during the fermenlation of glucose and maintenance of a pH below 4.5 in an old culture. Five drops of 0.04 % solution of methyl red are added to the culture in glucose phosphate medium incubated at 30°C for five days, mixed well and read at once. A red colour is positive while yellow signifies a negative test.

5. Voges-Proskauer test (VP): This test depends on the production of acetyle methylcarbional (CH_3. CHOH. CO. CH_3) from pyruvic acid, as an intermediate stage in its conversion to 2:3 butylene glycol. In the presence of alkali and atmospheric oxygen, the small amount of acetyl methyl carbionol present in the medium is oxidised to

diacetyl ($CH_3 . CO . CO . CH_3$) which reacts with the peptone of the broth to give a red colour.

The test is performed by adding 0.6 ml of a 5% solution of a-naphthol in ethanol and 0.2 ml of 40% KOH to one ml of a glucose phosphate culture of the organism incubated at 30°C for five days or 37°C for 48 hours. In a positive reaction, a pink colour appears in 2–5 minutes, deepening to magenta or crimson in half an hour. In a negative reaction, it remains colourless for half an hour. Traces of pink colouration should be neglected.

6. *Citrate utilisation:* Koser's citrate medium has citrate as the sole source of carbon. Ability to use this substance is indicated by the production of turbidity in the medium.

Indole, MR, VP and citrate tests are very useful in the identification and classification of enteric Gram negative bacteria. These tests are commonly referred to by the sigla 'IMViC' tests.

7. *Nitrate reduction:* This is tested after growing the bacterium for five days at 37°C in a broth containing 1% KNO_3. The test reagent consists of a mixture of equal volumes of solutions of sulphanilic acid and a-naphthylamine in 5 N acetic acid mixed just before use. 0.1 ml of the test reagent is added to the culture. A red colour developing within a few minutes is a positive test while absence of colour indicates a negative reaction. This is a test for the presence of the enzyme nitrate reductase which reduces nitrate to nitrite.

8. *Production of ammonia:* To a peptone water culture grown for five days at 37°C, Nessler's reagent is added. Brown colour is positive and faint yellow colour negative.

9. *Urease test:* This test is done in Christensen's urease medium. Inoculate the slope heavily and incubate at 37°C. Examine after four hours and after overnight incubation. The test should not be considered negative till after four days' incubation. Urease positive cultures produce a purple pink colour. Urease producing bacteria reduce urea to ammonia and hence the colour.

$$NH_2CO.NH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

10. *Hydrogen sulphide production:* Some organisms decompose sulphur-containing aminoacids producing H_2S among the products. When cultured in media containing lead acetate, they turn them black or brown. Instead of lead acetate, ferric ammonium citrate or ferrous acetate can be used. A very delicate method is to grow the organisms in culture tubes, inserting between the cotton plug and the tube a filter paper strip soaked in 10% lead acetate solution and dried. Browning or blackening of the paper indicates H_2S production.

11. *Methylene blue reduction:* One drop of 1% aqueous methylene blue is added to the broth culture, and incubated at 37°C. Complete decolourisation is strong positive while green colouration is weak positive.

12. *Catalase production:* Add a loopful of 10% H_2O_2 on colonies on nutrient agar. Prompt effervescence indicates catalase production. Cultures on media containing blood are unsuitable for the test as blood contains catalase.

13. *Oxidase reaction:* This reaction is believed to be due to a cytochrome oxidase which catalyses the oxidation of reduced cytochrome by molecular oxygen. A 1.0–1.5% solution of tetra methyl p-phenylene diamine hydrochloride is poured over the colonies. Oxidase positive colonies become maroon, purple and black in 10–30 minutes. This test can also be done by Kovac's method. A small strip of filter paper, soaked in the oxidase reagent, is laid in a Petri dish and the colony to be tested is smeared on the paper in a line about 5 mm long. In a positive reaction the smeared area turns dark in 10–60 seconds. The solution should be freshly prepared.

14. *Egg yolk reaction:* Organisms producing lecithinase during growth (e.g., *Cl. welchii*), when grown on a solid egg yolk medium, form colonies surrounded by a zone of clearing.

15. Growth in presence of KCN: Buffered peptone liquid medium containing KCN in a final concentration of about 1/13,000 is used to identify some enteric bacilli.

16. Composite media are increasingly being used for idenfitication of isolates. These are convenient and economical, as a single composite medium indicates different properties of the bacterium, which otherwise would have required the use of many separate media. A composite medium widely used is the Triple Sugar Iron (TSI) agar medium which indicates whether a bacterium ferments glucose only, or lactose and sucrose also, with or without gas formation, besides showing the production of H_2S. The medium is distributed in tubes with a butt and slant. After inoculation, if the slant remains red and the butt becomes yellow, all sugars — glucose, lactose and surcose — are fermented. Bubbles in the butt indicate gas production and blackening of the medium shows formation of H_2S. TSI medium facilitates the preliminary identification of Gram negative bacilli.

Other tests such as fermentation of organic acids, oxidation of gluconate, aminoacid decarboxylation, and hydrolysis of sodium hippurate are in use. With increasing knowledge of the metabolic processes in the growth of various bacteria, the tests too are on the increase. Special manuals have to be consulted for the details and utility of these tests.

Antigenic structure

By using specific sera we can identify organisms by agglutination reaction.

Bacteriophage and bacteriocin typing

Bacteriophage typing and bacteriocin typing enable intraspecies typing of some bacteria.

Pathogenicity

For pathogenicity tests, the commonly used laboratory animals are the guinea pig, rabbit, rat and mouse. The routes used may be subcutaneous, intramuscular, intraperitoneal, intracerebral or intravenous. The oral route or the nasal spray can also be used.

Identification of a bacterium is, therefore, an elaborate and time consuming process. It has to be done with meticulous care taking into consideration the sum total of the characteristics, without unduly weighting any single or very small group of characteristics.

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7 Bacterial Taxonomy

Bacterial taxonomy comprises three components:

1. classification, or the orderly arrangements of units,
2. identification of an unknown with a defined and named unit, and
3. nomenclature, or the naming of units.

For purposes of classification, it is necessary to determine as many characters of bacteria as possible. Such characters may be weighted, greater importance being given to some than to others, or they may be assigned equal importance, depending on the method employed for classification. On the contrary, for purposes of identifying bacterial isolates, it is important to devise a key using the minimum number of important characters which can be easily tested.

Bacterial classification

The first attempts at bacterial classification *(Mueller, 1786; Ehrenberg, 1838)* were made when little was known about bacteria. Haeckel (1866) classified all unicellular organisms as *Protista*. Cohn (1872–75) made a morphological classification, integrating bacteria with the blue-green algae in the class *Schizophyta*. A detailed system of classification was proposed by Migula (1894). As more information became available on the physiological and biochemical properties of bacteria, these were employed in proposing new systems of bacterial classification by Knight (1936), Kluyver and van Niel (1936) and others.

Bacterial classification presents special problems. Linnaeus (1735) divided all living beings into two kingdoms, plant and animal. Bacteria had been placed in the plant kingdom and designated *Schizomycetes* (fission fungi). But as bacteria present features common to both plants and animals, it has been proposed that a new kingdom, *Monera*, be created to accommodate all microorganisms without true nuclei, plastids and sexual reproduction (Stanier and van Niel, 1941). This proposal has not met with universal acceptance.

Kingdoms are divided successively into Divisions, Classes, Orders, Families, Tribes, Genera and Species. For example, the full taxonomical position of the typhoid bacillus is as follows.

Division	...	*Protophyta*
Class	...	*Schizomycetes*
Order	...	*Eubacteriales*
Family	..	*Enterobacteriaceae*
Tribe	...	*Salmonellae*
Genus	...	*Salmonella*
Species	...	*Salmonella typhi*

The species concept in bacteria: The species is the standard taxonomical unit in biology. With higher forms of life, a species unit constitutes a stage of evolution, with a characteristic morphology and is delimited by the failure of interbreeding outside the unit. But in bacteria, the species concept is vague and ill defined. Due to the absence of fossil-remains in bacteria, the evolutionary status of species cannot be established. Morphlogical differences are insufficient for the definition of bacterial species. The general absence of sexual reproduction in bacteria prevents the use of inbreeding as a test for differentiation between species.

In spite of these difficulties, the concept of species provides a convenient unit in bacterial taxonomy. Besides morphological features, criteria useful for definition of bacterial species are physiological, biochemical, antigenic and pathogenic properties. As 'species' is a genetic concept, definitive information can be obtained by comparison of the nucleotide base ratios, which are constant for any one species but may be different in different species. Genetic homology can be demonstrated by the binding exhibited between DNA isolated from different individuals of the same species.

An important difference between classification of bacteria and of higher organisms is that in the former, the properties of a population are studied, and not of an individual. A population derived by binary fission from a single cell is called a *clone*. A single bacterial colony represents a clone. Though all cells in a clone are expected to be identical in all respects, a few of them may show differences due to mutation. A population of bacteria derived from a particular source, such as a patient, is called a *strain*.

The general absence of sexual reproduction in bacteria serves to maintain their characters constant. But bacteria possess several features that contribute to a varying degree of heterogenicity in their populations. Their short generation time and high rate of mutation lead to the presence, in any population, of cells with altered characters. Methods of genetic exchange such as transformation, transduction and conjugation cause differences in characters. Prophage and plasmid DNA can induce new properties.

Phylogenetic classification: There are two approaches to bacterial classification. The hierarchical classification represents a branching tree-like arrangement, one character being employed for division at each branch or level. This system is called *phylogenetic* because it implies an evolutionary arrangement of species. Here some characters are arbitrarily given special weightage. Depending on the character so chosen, the classification would give different patterns. For example, the intestinal Gram negative bacilli have been traditionally classified depending on whether they ferment lactose or not. While this provides a useful distinction between the pathogenic and nonpathogenic groups of these bacilli, a somewhat different but still useful arrangement would be obtained if fermentation of sucrose is the criterion used instead. While classification based on weighted characters is a convenient method, it has the serious drawback that the character used may not be valid. Fermentation of lactose, in the example cited, is not an essential and permanent character. It may be acquired or lost, upsetting the system of arrangement.

The Adansonian classification: The Adansonian classification, so called after Michael Adanson who introduced it in the 18th century, avoids the use of weighted characters. It makes no phylogenetic assumption, but merely takes into account all the characters expressed at the moment of study. Hence it is called a *phenetic system*. It gives equal weight to all measurable features, and groups organisms on the basis of similarities in large numbers of characters. The availability of computers has extended the scope of phenetic classification by permitting comparisons of very large numbers of properties of several organisms at the same time. This is known as *numerical taxonomy*.

Molecular or genetic classification: This is based on the degree of genetic relatedness of different organisms. Since all properties are ultimately based on the genes present, this classification is said to be the most natural or fundamental method. DNA relatedness can be tested by studying the nucleotide sequences of DNA and by DNA hybridisation or recombination methods. The nucleotide base composition and base ratio (Adenine-Thymine: Guanine-Cytosine ratio) varies widely among different groups of microorganisms, though it is constant for members of the same species. Molecular classification has been employed more with viruses than with bacteria.

No method of bacterial classification is universally accepted. The method most widely adopted is presented in the successive editions of *Bergey's Manual of Determinative Bacteriology.*

Intraspecies classification: For diagnostic or epidemiological purposes, it is often necessary to subclassify bacterial species. This may be based on biochemical properties (biotypes), antigenic features (serotypes), bacteriophage susceptibility (phage types) or production of bacteriocins (colicin types). A species may be divided first into groups and then into types, as for example, in streptococci.

Nomenclature

The need for applying generally accepted names for bacterial species is self-evident. Chaos will result if the same bacterium is referred to by different names by different workers. International agreement on bacterial nomenclature is ensured by the Code of Bacteriology Nomenclature which has the authority of the International Association of Microbiological Societies.

Two kinds of names are given to bacteria. The first is the *casual* or common name which varies from country to country and is in the local language. Names such as 'typhoid bacillus' and 'gonococcus' are casual names. Such names are useful for communication at the local level. The second is the *scientific* or international name which is the same throughout the world. The scientific name consists usually of two words, the first being the name of the genus and the second the specific epithet (e.g., *Bacillus subtilis)*. The generic name is considered a Latin noun. The specific epithet is an adjective or noun and indicates some property of the species (e.g., *albus,* meaning white), the animal in which it is found (e.g., *suis,* meaning pig), the disease it causes (e.g., *tetani,* of tetanus), the person who discovered it (e.g., welchii, after *Welch)* or the place of its isolation (e.g., london). The generic name always begins with a capital letter and the specific epithet with a small letter, even if it refers to a person or place (e.g., *Salmonella london*).

Type cultures

As a point of reference, type cultures of bacteria are maintained in International Reference Laboratories. The type cultures will contain representatives of all established species. The original cultures of any new species described will be deposited in type collections. They will be made available by the Reference Laboratories to other workers for study and comparison

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8 Bacterial Genetics

Genetics is the study of heredity and variation, seeking to understand 'the causes' of the resemblances and differences between parents and their progeny. Like other organisms, bacteria also breed true and maintain their characters constant from generation to generation, yet, at the same time, exhibit variations in particular properties in a small proportion of their progeny. Though heritability and variation in bacteria had been noticed from the early days of bacteriology, it was not realised then that bacteria too obey the laws of genetics. Even the existence of a bacterial nucleus was a matter of controversy. The differences in morphology and other properties were attributed by Nägeli (1877) to bacterial *pleomorphism*, which postulated the existence of but a single or, perhaps, a few species of bacteria, which possessed a protean capacity for variation. With the development and application of precise methods of pure culture, it became apparent that different types of bacteria retained constant form and function through successive generations. This led to the opposite concept of *monomorphism*, proposed by Cohn and Koch, which admitted of little potential for variation and separated bacteria into species upon single character differences.

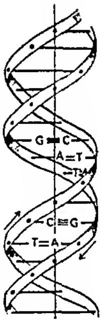

Fig 8 1 DNA double helix

It is only since the 1940's that principles of genetics were applied to bacteria and their viruses. This has led not merely to a better understanding of the genetic processes, but also to fundamental advances in biology and biochemistry and to the birth of a new branch of science, molecular biology.

Basic principle of molecular biology

The 'central dogma' of molecular biology is that deoxyribonucleic acid (DNA) carries genetic information, which is transcribed on to ribonucleic acid (RNA) and then translated as the particular polypeptide (DNA → RNA → Polypeptide). As the nature and functions of a cell are basically determined by the specific polypeptides that constitute its proteins and enzymes, it is evident that the essential material of heredity is DNA which is the storehouse of all information for protein synthesis. (An exception

exists in the case of some viruses in which the genetic material is RNA instead of DNA).

The DNA molecule is composed of two chains of nucleotides wound together in the form of a 'double helix' (Fig. 8.1). Each chain has a backbone of deoxyribose and phosphate residues arranged alternately. Attached to each deoxyribose is one of four nitrogenous bases, the purines adenine (A) and guanine (G), and the pyrimidines thymine (T) and cytosine (C). The doublestranded nature of the molecule is stabilised by hydrogen bonding between the bases on the opposite strands in such a manner that adenine is always linked to thymine, and guanine to cytosine (Fig. 8.2).

Adenine and thymine thus form a complementary base pair, and guanine and cytosine form another. A molecule of DNA will, therefore, contain as many units of adenine as thymine, and of guanine as cytosine, but the ratio of each pair of bases (A + T)/(G + C), though constant for each species, varies widely from one bacterial species to another. The DNA molecule replicates by first unwinding at one end to form a fork, each strand of the fork acting as a template for the synthesis of a complementary strand, with which it then forms a double helix.

Basically, RNA is structurally similar to DNA, except for two major differences. It contains the sugar ribose (instead of deoxyribose present in DNA) and the base uracil (instead of thymine present in DNA). Three distinct types of RNA can be distinguished on the basis of structure and function: messenger RNA (m-RNA), ribosomal RNA and transfer RNA (t-RNA). DNA acts as the template for the synthesis of m-RNA and, therefore, the bases in the two will be complementary to each other. Adenine, guanine, cytosine and uracil in m-RNA will be, respectively, complementary to thymine, cytosine, guanine and adenine in the DNA.

Genetic information is stored in the DNA as a code, the unit of the code (codon) consisting of a sequence of three bases, i.e., the code is triplet Each codon specifies for a single aminnacid, but the code is 'degenerate' so that more than one codon may exist for the same aminnacid. Thus, the triplet AGA codes for arginine, but the trip-

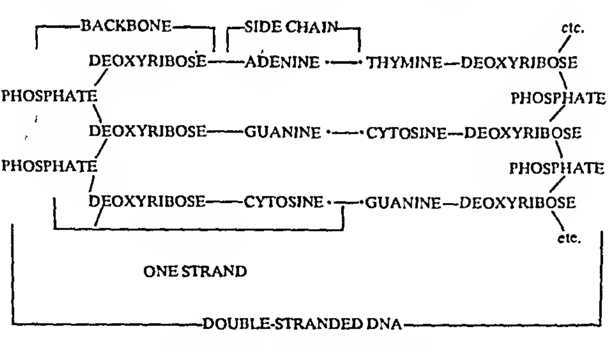

Fig. 8.2 A segment of double stranded DNA illustrating its chemical structure

lets AGG, CGU, CGC, CGA and CGG also code for the same aminoacid. The code is non-overlapping, each triplet being a distinct entity, and no base in one codon is employed as part of the message of an adjacent codon. Three codons (UAA, UAG and UGA) do not code for any aminoacid and are called 'nonsense codons'. They act as punctuation marks (stop codons) terminating the message for the synthesis of a polypeptide. A segment of DNA carrying codons specifying for a particular polypeptide is called a 'cistron' or gene. A DNA molecule consists of large number of cistrons, each of which contains hundreds of thousands of nucleotides. The bacterial chromosome consists of a double-stranded molecule of DNA arranged in a circular form. When straightened, it is about 1000μ in length. It carries, on an average, about 1000–3000 cistrons.

In higher forms of life, several stretches of DNA that do not appear to function as codons occur between the coding sequences of genes. These apparently useless noncoding intrusions are called *introns*, while the stretches of coded genes are called *exons*. During transcription, the genome is copied in its entirety, both introns and exons. The introns are then excised from the RNA copy before being translated by the ribosomes into proteins

Extrachromosomal genetic elements

Besides the chromosomal DNA, bacteria may also possess extrachromosomal genetic elements. Two terms have been used for describing these structures. They are called *plasmids* when they consist of DNA situated in the cytoplasm in the free state and reproducing autonomously (independent replicons). Genetic elements that exist in one of two alternate states, either autonomously in the cytoplasm, or in the integrated state, attached to the bacterial chromosomes are called *episomes*. Differentiation between plasmids and episomes is often not possible and the two terms are frequently used synonymously.

Plasmids and episomes are not essential for the normal life and function of the host bacterium, though they may confer on it properties such as drug resistance and toxigenicity conferring survival advantage under appropriate conditions.

Some plasmids endow the host cell with 'maleness' or the ability to conjugate with other cells. Following conjugation, the recipient acquires the plasmid, becoming a 'male' or 'donor' cell in the process. The basis of maleness is the ability to form special pili (sex pili) which are coded for by the plasmid. Such plasmids are called transmissible plasmids. An example is the drug resistance (R) factor in enterobacteria. Some other plasmids are not transmissible by conjugation, but are conveyed from cell to cell through the agency of bacteriophages (transduction). The penicillinase plasmid which induces penicillin resistance in staphylococci is an example of a transducible plasmid.

Technological advances in molecular biology have made it possible to introduce genes from eukaryotic cells into plasmids, which replicate stably in *E. coli*. Such recombinant DNA molecules form the basis of genetic engineering techniques.

Genotypic and phenotypic variations

The sum total of genes the make up the genetic apparatus of a cell establishes its *genotype*, which is the hereditary constitution of the cell that is transmitted to its progeny. The genotype includes the complete genetic potential of the cell, all of which may or may not be expressed in a given environmental situation.

The *phenotype* ('phaeno': display) is the physical expression of the genotype in a given environment. It follows, therefore, that a cell may exhibit different phenotypic appearances in different situations; for example, the typhoid bacillus is normally flagellated, but when grown in phenol agar, the flagella are not synthesised. This is only a phenotypic variation determined by the environment and is reversed when subcultured from phenol agar into broth. Another example

lac Z *lac Y* *lac A*

Regulator Promotor Operator. Structural genes (*lac Operon*)

of environmental influence is the synthesis by *E. coli* of the enzyme beta-galactosidase, necessary for lactose fermentation. The bacillus possesses the genetic information for the synthesis of the enzyme, but the actual synthesis takes place only when it is grown in a medium containing lactose. When grown in a medium containing glucose only, the enzyme is not synthesised. Such enzymes which are synthesised only when induced by the substrate are called *induced enzymes*, as opposed to *constitutive enzymes*, which are synthesised irrespective of the presence or absence of the substrate.

The regulation of enzyme induction illustrates the economy of nature, ensuring that such enzymes are synthesised only when the appropriate substrates are present. At other times, the enzyme synthesis is shut off or 'repressed'. Studies by Jacob and Monod on the synthesis of beta-galactosidase by *E. coli* led to the 'operon' concept, which explains this regulatory mechanism involving two types of genes, structural and regulatory. Genes determining the structure of a particular protein are called 'structural genes'. The activity of structural genes is controlled by 'regulator genes' which lie adjacent to them.

Lactose fermentation by *E. coli* requires three enzymes, beta-galactosidase, galactoside permease and transacetylase, coded for respectively by the structural genes *lac* Z, *lac* Y and *lac* A arranged linearly in sequence, forming a functional unit known as the *lac operon*. The regulator gene in this case is *lac I*, which codes for a 'repressor', an allosteric protein molecule which can combine either with the 'operator' region on the chromosome, or with the inducer. Between *lac I* and the structural *lac* genes lie the promotor and the operator regions. For transcription of RNA for the enzyme synthesis, the RNA polymerase has to attach to the promotor region and travel along the structural genes in sequence. The operator region lies between the promotor and the structural genes. The sequence of genes is indicated above.

In the resting stage, the repressor molecule is bound to the operator, preventing the passage of RNA polymerase from the promotor to the operon. The repressor molecule has an affinity for lactose, in the presence of which it leaves the operator region free, enabling the transcription to take place. Lactose thus acts both as the inducer and the substrate for beta-galactosidase. When the lactose present is completely metabolised, the repressor again attaches to the operator, switching off transcription.

Phenotypic variations are influenced by the environment, limited in range by the genotype, temporary and not heritable. Variations are said to be genotypic when they are due to alterations in the genome. Genotypic variations are stable, heritable and not influenced by the environment. They may occur by mutation, or by one of the mechanisms of genetic transfer or exchange, such as transformation, transduction, lysogenic conversion and conjugation.

Mutation

Mutation is a random, undirected, heritable variation caused by an alteration in the nucleotide sequence at some point of the DNA of the cell. It may be due to addition, deletion or substitution of one or more bases. A *missense mutation* is one in which the triplet code is altered so as to specify an aminoacid different from that normally located at a particualr position in the protein. Deletion of a nucleotide within a gene may cause premature polypeptide chain termination by generating a nonsense codon, i.e., *nonsense mutation*. *Transversion* is substitution of a purine

for a pyramidine and vice versa in base pairing. *Suppressor mutation* is reversal nf a mutant phenotype by another mutation at a position on the DNA distinct from that of the original mutation. All genes are susceptible to mutational events, but not all mutations are expressed. Some mutations involve vital functions, and such mutants are nonviable *(lethal mutation)*. A type of lethal mutation which is of great interest is 'conditional mutation'. A *conditional lethal mutant* may be able to live under certain conditions (permissive conditions), hut not under others (nonpermissive or restrictive conditions). The commonest type of conditional mutant is the temperature-sensitive *(ts)* mutant, which is able to live at the permissive temperature (say 35°C), but not at the restrictive temperature (say 39°C).

Each gene undergoes mutation with a fixed frequency. Mutation rates of individual genes in bacteria range from 10^{-2} to 10^{-10} per bacterium per division The molecular mechanism of mutation is that during DNA replication, some 'error' creeps in while the progeny strands are copied For instance, instead of thymine bonding with adenine, it may, due to tautomerism, sometimes bond with guanine Though mutation occurs spontaneously, its frequency can be increased by several agents (mutagens), such as UV rays, alkylating agents, acridine dyes, 5-bromouracil and 2-aminopurine.

Mutation is a natural event, taking place all the time, at its particular frequency in all dividing forms of life. Most mutants, however, go unrecognised as the mutation may be lethal nr it may affect some minor function that may not be expressed. Mutation is best appreciated when it involves a function which can be readily observed. For example, an *E. coli* mutant that loses its ability to ferment lactose can be readily detected on MacConkey agar, but is unrecognisable on nutrient agar Mutation is of vital importance when it confers a survival advantage. If a streptomycin resistant mutant of the tubercle bacillus develops in a patient under treatment with the drug, it multiplies selectively and ultimately replaces the original drug sensitive population of bacteria. But in a patient who is not on treatment, the mutation confers nn survival advantage and, therefore, preferential multiplication of the mutant does not occur. Such changes in the character of bacterial populations, observed in the presence of a selective environment, were formerly considered to be 'adaptations'. By a *post hoc, ergo propter hoc* reasoning, the environment and the variation were believed to have a cause and effect relationship. Such induced variations were considered heritable in the Lamarckian sense. It was the demolition of this concept of adaptation in the 1940's that established bacterial genetics on a firm scientific basis

The proof that bacteria undergo spontaneous mutation independent of the environment was first provided by Luria and Delbruck (1943) by the 'fluctuation test'. They found that very wide fluctuations occurred in the numbers of bacteriophage resistant *E. coli* colonies when samples were plated from several separate small volume cultures, as compared to samples tested from a single large volume culture. Statistically this indicated that mutations occurred randomly in the separate small volume cultures, some early and some late, resulting in the wide fluctuation. In the large volume culture, fluctuations were within limits of sampling error. However, the logic of this experiment was not widely appreciated by microbiologists, probably due to the statistical interpretation. It was the simple, but elegant 'replica-plating' technique of Lederberg and Lederberg (1952) that proved the point beyond doubt. Using a velvet template, they were able to transfer inocula from colonies on a master plate, on to a number of other plates, retaining the relative positions of the colonies in all the plates. By such replica-plating on culture plates with and without bacteriophages, they were able to show that bacteriophage resistant mutants appeared without the bacteria ever having had contact with the selective agent.

Mutation may affect any gene and hence may modify any character of the bacterium. Mutants may vary in properties such as nutritional requirements, biochemical reaction, antigenic

structure, morphological features, colony form, drug susceptibility, virulence and host range. The practical importance of hacterial mutation lies mainly in the field of drug resistance and the development of live vaccines.

Tranmission of genetic material

Transformation: Transformation is the transfer of genetic information through the agency of free DNA. It was the first example of genetic exchange in bacteria to have been discovered. Griffith in 1928 found that mice died when injected with a mixture of live noncapsulated (R) pneumonococci and heat-killed capsulated(S) pneumococci, neither of which separately proved fatal. If in the experiment, the live(R) pneumococci were derived from capsular type II and the killed (S) strain from type I, from blood cultures of mice that died, the live type I capsulated pneumococcus could be isolated, showing that some factor in the heat-killed type I pneumococcus had transferred the information for capsule synthesis to the rough strain. Such transformation was subsequently demonstrated *in vitro* also. The nature of the transforming principle was identified as DNA by Avery, Macleod and McCarty in 1944.

Transformation has been studied mainly in pneumococci, *Bacillus* species and *Haemophilus influenzae*, though it may occur in other bacteria also. Any character may be transferred by transformation. Pieces of DNA involved in transformation may carry about 10–50 genes. The frequency and significance of transformation in bacteria under natural conditions are not known.

Transduction: The transfer of a portion of the DNA from one bacterium to another by bacteriophage is known as transduction. Bacteriophages are viruses that parasitise bacteria and consist of a nucleic acid core and a protein coat. During assembly of hacteriophage progeny inside infected bacteria, 'packaging errors' may happen occasionally. A phage particle may have at its core, besides its own nucleic acid, a segment of the host DNA. When this particle infects another bacterium, DNA transfer is effected and the recipient cell acquires new characters coded by the donor DNA. Transduction may he 'generalised', when it involves any segment of the donor DNA at random, or it may be 'restricted', when a specific bacteriophage transduces only a particular genetic trait. Restricted transduction has been studied intensively in the 'lambda' phage of *E. coli*. The prophage lambda is inserted in the bacterial chromosome always between the genes determining glactose utilisation *(gal)* and biotin synthesis *(bio)* and, therefore, it transduces only either of these.

Transduction is not confined to transfer of chromosomal DNA. Episomes and plasmids may also be transduced. The plasmids determining penicillin resistance in staphylococci are transferred from cell to cell by transduction.

Transduction appears to be the most widespread mechanism of gene transfer among prokaryotes and provides an excellent tool for the genetic mapping of bacteria. Any group of hacteria for which bacteriophages exist, can be subject to transduction. It has heen reported that transduction may occasionally be effected in eukaryotic cells also. Transduction has been proposed as a method of genetic engineering in the treatment of some inborn errors of metabolism. Claims have been made that the metabolic defects in fibroblasts from galactosaemic patients can be corrected by transduction using the lambda phage carrying the *gal* gene.

Lysogenic conversion: Bacteriophages exhibit two types of life cycle. In the *virulent* or *lytic* cycle, large numbers of progency phages are built up inside the host bacterium, which ruptures to release them. In the *temperate* or *nonlytic* cycle, the host hacterium is unharmed. The phage DNA hecomes integrated with the bacterial chromosome as the *prophage,* which multiplies synchronously with the host DNA and is transferred to the daughter cells. This process is

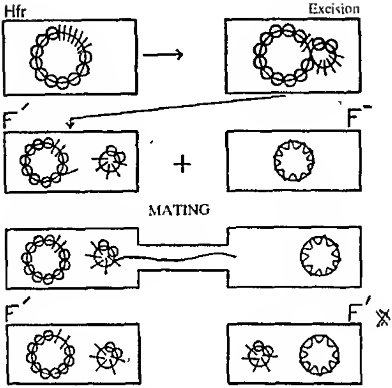

Fig 8.3 Sexduction. The integrated *F* factor of an *Hfr* cell may revert to the cytoplasmic state. During excision, some host genes may be incorporated in the *F* factor (F'). When an F' cell mates with an F^- cell, the host gene is transferred to the recipient

called *lysogeny* and bacteria harbouring prophages are called *lysogenic* bacteria. Lysogeny is extremely frequent in nature.

In lysogenic bacteria, the prophage behaves as an additional segment of the bacterial chromosomes, coding for new characters. This process by which the prophage DNA confers genetic information to a bacterium is called *lysogenic* or *phage conversion*. In transduction, the phage acts only as a vehicle carrying bacterial genes from one cell to another, but in lysogenic conversion the phage DNA itself is the new genetic element. Lysogenic conversion influences susceptibility to bacteriophages (immunity to superinfection with the same or related phages), and antigenic characters. Of great medical importance is the lysogenic conversion in diphtheria bacilli, which acquire toxigenicity (and therefore virulence) by lysogenisation with the phage beta. Elimination of the phage from a toxigenic strain renders it nontoxigenic.

Conjugation: Conjugation is the process whereby a 'male' or 'donor' bacterium 'mates' or makes physical contact with a 'female' or 'recipient' bacterium and transfers genetic elements into it. This has been considered to be the bacterial equivalent of sexual mating in higher organisms, but the analogy is artificial as, following conjugation, the female bacterium is in turn converted into a male cell! Bacterial conjugation was first described by Lederberg and Tatum (1946) in a strain of *E. coli* called K12 and has been most extensively studied in this strain.

Conjugation takes place between a male cell and a female cell. The maleness or donor status of

a cell is determined by the presence in it of a plasmid which codes for specialised fimbria (sex pilus) which projects from the surface of the cell. The plasmid DNA replicates and a copy of it passes from the donor to the recipient cell, probably along the sex pilus (conjugation tube). As a result, the recipient attains donor status and can in turn conjugate with other female cells. The maleness in bacteria is thus a transmissible or 'infectious' character. Along with the plasmid DNA, portions of the host DNA also are sometimes transferred to the recipient. The donor DNA then combines with the DNA of the recipient, effecting genetic recombination. It was in *E. coli* K12 that the role of plasmids in conjugation was first recognised. The plasmid responsible was termed the 'sex factor' or 'fertility (F) factor'. When other similar plasmids were also discovered, the name 'transfer factor' came to be used for all such plasmids which conferred on their host cells the ability to act as donors in conjugation.

The F factor: The *F* factor is a transfer factor that contains the genetic information necessary for the synthesis of the sex pilus and for self-transfer, but is devoid of other identifiable genetic markers such as drug resistance. Cells carrying the *F* factor (F + cells) have no distinguishing features other than their ability to mate with F– cells and render them F+. The *F* factor is actually an episome and has the ability to exist in some cells in the 'integrated state' or inserted into the host chromosome. Such cells are able to transfer chromosomal genes to recipient cells with high frequency and are known as *Hfr* cells. Following conjugation with an *Hfr* cell, an F– only rarely becomes F+, though it receives chromosomal genes from the donor.

This conversion of an F+ cell into the *Hfr* state is reversible. When the *F* factor reverts from the integrated to the free state, it may sometimes carry with it some chromosomal genes from near to its site of attachment. Such an F factor incorporating some chromosomal genes is called an F prime *(F')* factor. When an F' cell mates with a recipient, it transfers, along with the *F* factor, the host genes incorporated with it. This process of transfer of host genes through the *F* factor resembles transduction and has, therefore, been called *sexduction* (Fig. 8.3).

Colicinogenic (Col)-factor: Several strains of coliform bacteria produce colicins — antibiotic substances which are specifically and selectively lethal to other enterobacteria. As similar substances are produced by bacteria other than coliforms also (pyocin by *Pseudomonas pyocyanea*, diphthericin by *Corynebacterium diphtherae)*, the name *bacteriocin* has been given to this group of substances. Chemically, bacteriocins vary in composition from proteins, to lipoprotein-polysaccharide complex, to a particle resembling a defective phage. The specificity of action of bacteriocins enables intraspecies classification of certain bacteria (e.g., *Shigella sonnei, Ps. pyocyaneus)*.

Colicin production is determined by a plasmid called the *Col* factor, which resembles the *F* factor in promoting conjugation, leading to self-transfer and, at times, transfer of chromosomal segments.

Resistance transfer factor (RTF): This plasmid is of great medical importance as it is responsible for the spread of multiple drug resistance among bacteria.

This extrachromosomal mechanism of drug resistance was first reported by Japanese workers (1959) investigating the sudden increase in infections caused by shigella strains resistant simultaneously to sulphonamides, streptomycin, chloramphenicol and tetracycline. They observed that patients excreting such shigella strains also shed in their faeces *E. coli* strains resistant to the same drugs. Transfer of multiple drug resistance was demonstrated between *E. coli* and shigella strains both *in vitro* and *in vivo*. The resistance is plasmid mediated and is transferred by conjugation. This mechanism of drug resistance is known as *transferable, episomal or infectious drug resistance*.

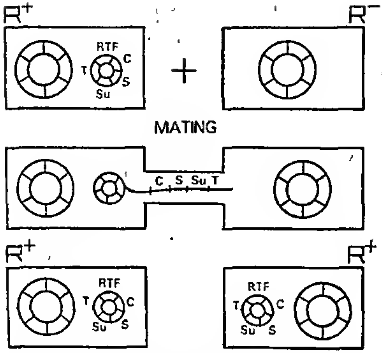

Fig 8.4 Transferable drug resistance The R$^+$ cell carries the R factor, consisting of RTF and r-determinants Its transfer to a sensitive R$^-$ bacterium, converts the recipient into a resistant R$^+$ cell

This plasmid consists of two components — the transfer factor called the *resistance transfer factor (RTF)* which is responsible for conjugational transfer, and the *resistance determinant (r)* for each of the several drugs. The whole plasmid (*RTF*+r determinants) is known as the *R* factor. An *R* factor can have several *r* determinants, and resistance to as many as eight or more drugs can be transferred simultaneously (Fig. 8.4). Sometimes the *RTF* may dissociate from the *r* determinants, the two components existing as separate plasmids. In such cases, though the host cell remains drug resistant, the resistance is not transferable. The *RTF* can have attached to it determinants other than those for drug resistance. Enterotoxin and haemolysin production in some enteropathogenic *E. coli* have been shown to be transmitted by this transfer factor.

Transferable drug resistance is seen widely in various pathogenic and commensal bacteria of man and animals — *Enterobacteriaceae, Vibrio, Pseudomonas, Pasteurella.* The transfer can be effected readily *in vitro*, but in the normal gut, it is inhibited by several factors such as anaerobic conditions, bile salts, alkaline pH and the abundance of anaerobic, Gram positive bacteria minimising the chances of contact between donor and suitable recepient cells. But in the intestines of persons on oral antibiotic therapy, transfer occurs readily due to the destruction of the sensitive normal flora and the selection pressure produced by the drug.

Transferable drug resistance is now universal in distribution and involves all antibiotics in common use. Its incidence is directly proportional to the frequency of use of antibiotics in the area. Bacteria carrying *R* factors can spread from animals to man. Hence indiscriminate use of antibiotics in veterinary practice or in animal feeds can also lead to an increase of multiple drug resis-

tance in the community. The addition of antibiotics in animal feeds has for this reason been prohibited by legislation in some countries.

Genetic mechanisms of drug resistance in bacteria

Bacteria may acquire drug resistance by mutation, or by one of the methods of genetic transfer. The biochemical mechanisms of resistance may be several, including decreased permeability to the drug, development of alternate metabolic pathways, and production of enzymes inactivating the drugs.

Mutational resistance is mainly of two types:

1. the step-wise mutation, as seen with penicillin, where high levels of resistance are achieved only by a series of small-step mutations, and

2. the 'one-step' mutation, as seen with streptomycin, where the mutants differ widely in the degree of resistance, some exhibiting low resistance, while others may be highly resistant, and some even streptomycin dependent.

In clinical practice, mutational resistance is of great importance in tuberculosis. If a patient is treated with streptomycin alone, initially the bacilli die in large numbers but, soon, resistant mutants appear and will multiply unchecked. If two or more antituberculous drugs are used for combined treatment, repopulation by resistant mutants does not occur, as a mutant resistant to one drug will be destroyed by the other drug. The possibility of a mutant exhibiting resistance to multiple drugs simultaneously is so remote as to be virtually nil. This is the rationale behind combined treatment in tuberculosis.

Resistance transfer by transformation can be demonstrated experimentally, but its significance in nature is not known. Acquisition of resistance by transduction is common in staphylococci. The penicillinase plasmids, which are transmitted by transduction, may also carry determinants for resistance to mercuric chloride and erythromycin.

Transferable drug resistance mediated by the *R* factor is the most important method of drug resistance. Acquisition of an *R* factor simultaneously confers resistance against several drugs and, therefore, treatment with a combination of drugs is of no avail. The mechanism of resistance is by the production of degrading enzymes, and

TABLE 8.1
Comparison of mutational and transferable types of drug resistance in bacteria

	Mutational drug resistance	Transferable drug resistance
1	Involves resistance to one drug at a time.	Simultaneous resistance to multiple drugs. May involve all drugs used in clinical treatment.
2	Degree of resistance usually low.	Degree of resistance usually high.
3	May be overcome by high drug dosage.	High drug dosage ineffective.
4	Can be prevented by treatment with combination drugs	Combination of drugs cannot prevent.
5	Resistance does not spread.	Resistance spreads to other cells of same or different species of the genus.
6	Resistant mutants are usually metabolically defective.	Metabolically normal.
7	Virulence may be low.	Virulence not decreased

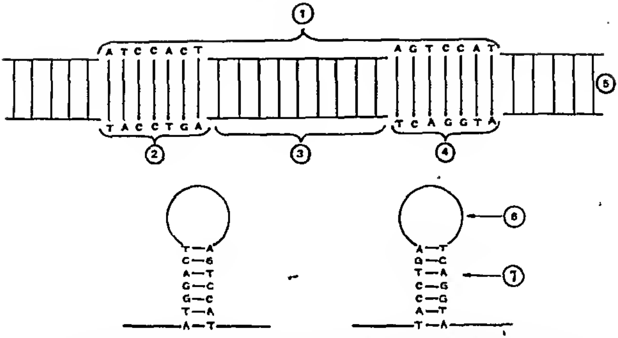

Fig 8.5 Diagrammatic representation of a transposon The upper figure shows a transposon along the course of a DNA molecule, consisting of a gene in the middle and inverted repeat sequences of nucleotides at either end The lower figures represent the stem and loop structure formed by each strand of the transposon The loop consists of the gene and the stem is formed by hydrogen bonding between the terminal repeat sequences The stem and loop form can attach to insertion sites on recipient DNA 1 Transposon 2 Inverted repeat sequences. 3 Gene. 4 Inverted repeat sequences 5 Double stranded DNA. 6 Single stranded loop consisting of gene 7 Double stranded stem formed by bonding of terminal inverted sequences

the level of resistance is usually of a high order. Resistance may be transferred between bacteria of different taxonomic groups. While the resistant mutants usually have a lower growth rate and reduced virulence as compared to the wild strains, bacteria carrying *R* factors are apparently normal in other respects. There is evidence that in some instances, *R* factors may even lead to enhanced virulence. Multiple drug resistance was initially seen in bacteria causing diarrhoeas and such mild infections that did not warrant antibiotic treatment as a routine. It has subsequently spread to involve various Gram negative bacilli that cause urinary and systemic infections Most disturbing has been the acquisition of such resistance by typhoid bacilli in some endemic areas.

In the laboratory, *R* factors may sometimes be eliminated by treating bacteria with acridine dyes or ethidium bromide. But in the community, the only way to prevent widespread dissemination of multiple resistance is to restrict the use of antibiotics to the essential minimum.

Transposable genetic elements

Certain structurally and genetically discrete segments of DNA have been identified that have the ability to move around between chromosomal and extrachromosomal DNA molecules within cells. These DNA molecules are called *transposons* and this mode of genetic transfer, *transposition*. A transposon is a segment of DNA with one or more genes in the centre, and the two ends carrying 'inverted repeat' sequences of nucleotides — nucleotide sequences complementary to each other, but in the reverse order. Because of this feature, each strand of the transposon can form a

single stranded loop carrying the gene, and a double stranded stem formed by hydrogen bonding between the terminal inverted repeat sequences (Fig. 8.5). Transposons get attached at certain regions of chromosomal, plasmid or phage DNA. Insertion of a transposon leads to the acquisition of new characters by the recipient DNA molecule.

By transposition, a segment of DNA can be transferred from one molecule to another molecule that has no genetic homology with either the transposable element or with the donor DNA. In this it differs from recombination. As sizeable chunks of DNA are added by transposition, the recipient molecule becomes heavier.

Characters transferred by transposons may sometimes confer survival advantage under appropriate environmental conditions. It has been suggested that the resistance-determinant segments of the R factors may have evolved as collections of transposons, each carrying a gene that confers resistance to one or several antibiotics.

Transposition is a mechanism for amplifying genetic transfers in nature and has been identified in microorganisms, plants and animals. Transposons appear to accomplish in nature, gene manipulations similar to the laboratory manipulations that have been called 'genetic engineering'.

Genetic engineering

It is now possible to isolate the genes coding for any desired protein from microorganisms or from cells of higher forms of life including man, and introduce them into suitable microorganisms, in which the genes would be functional, directing the production of the specific protein. This is known as the *Recombinant-DNA technology* or *Genetic engineering*. Such cloning of genes in microorganisms enables the production of any desired protein in pure form, in large quantities and at reasonable cost.

Different strategies have been employed for obtaining the desired genes. For very small proteins, such as the pituitary hormone somatostatin whose complete aminoacid sequences are known, the genes can be synthesised in the laboratory. With larger proteins, this is not possible. The DNA can be cleaved by specific enzymes called *restriction endonucleases* and the fragments containing the desired genes isolated. This does not work with DNA of higher organisms, as they contain introns. In such cases, the concerned messenger RNA can be isolated from cells producing the desired protein. A DNA copy is made from the mRNA using the enzyme reverse transcriptase. The double stranded DNA gene is then prepared using DNA polymerase. This is incorporated into suitable *vectors* or *carriers*, such as plasmids or temperate bacteriophages for insertion into microorganisms. The microorganism commonly employed is *Escherichia coli* K12, though many other bacteria and yeasts have also been used.

Genetic engineering has become an established branch of biotechnology with great scope for commercial exploitation. Cloned human insulin, interferons, somatostatin, growth hormones and many other biologicals have already been marketed. Safer vaccines can be produced by cloning the protective antigens of pathogens, as has already been done, for example, with foot and mouth disease, hepatitis B and rabies viruses. The versatile technique has many extramedical applications also.

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Part II

9 Infection

Infection and immunity involve interactions between the animal body (host) and the infecting microorganism. Based on their relationship to hosts, microorganisms can be classified as *saprophytes* (from G. *sapros*, decayed: and *phyton*, plant) and *parasites*. Saprophytes are free living microbes that subsist on dead or decaying organic matter. They are found in soil and water and play an important role in the degradation of organic materials in nature. They are generally incapable of multiplying on living tissues and, therefore, are of little relevance in infectious disease. Exceptionally, however, some saprophytes, for example *B. subtilis*, may infect devitalised hosts whose natural resistance is greatly reduced (opportunistic infection). Parasites are microbes that can establish themselves and multiply in hosts. Parasitic microbes may be either *pathogens* (from G. *pathos*, suffering; and *gen*, produce, i.e., disease-producing) or *commensals* (from L. *con*, with; and *mensa*, table, i.e., living together). Pathogens are microorganisms that are capable of producing disease in the host. Commensal microbes live in complete harmony with the host without causing any damage to it. The normal bacterial flora of the body consist largely of commensals. Many commensals behave as facultative pathogens in that they can produce disease when the host resistance is lowered.

It is necessary to distinguish between the term 'infection' and 'infectious disease'. The lodgement and multiplication of a parasite in or on the tissues of a host constitute infection. It does not invariably result in disease. In fact, disease is but a rare consequence of infection, which is a common natural event.

Infections may be classified in various ways. Initial infection with a parasite in a host is termed *primary infection*. Subsequent infections by the same parasite in the host are termed *reinfections*. When in a host whose resistance is lowered by a preexisting infectious disease, a new parasite sets up an infection, this is termed *secondary infection*. The term *focal infection* (more appropriately *focal sepsis*) indicates a condition where, due to infection or sepsis at localised sites such as appendix or tonsils, generalised effects are produced. When in a patient already suffering from a disease, a new infection is set up from another host or another external source, it is termed *cross infection*. Cross infections occurring in hospitals are called *nosocomial infections* (from G. *nosocomion*, hospital). The term *iatrogenic infection* refers to physician-induced infections resulting from investigative, therapeutic or other procedures. Depending on whether the source of infection is from the host's own body or from external sources, infections are classified as *endogenous* or *exogenous*, respectively. Based on the clinical effects of infections, they may be classified into different varieties. *Inapparent* infection is one where clinical effects are not apparent. The term *subclinical* infection is often used as a synonym. *Atypical infection* is one in which the typical or characteristic clinical manifestations of the particular infectious disease are not present. Some parasites, following infection, may remain in the tissues in a latent or hidden form proliferating

and producing clinical disease when the host resistance is lowered. This is termed *latent infection.*

Sources of Infection In man

Man: The commonest source of infection for man is man himself. The parasite may originate from a patient or a carrier. A *carrier* is a person who harbours the pathogenic microorganisms without suffering from any ill effect from it. Several types of carriers have been identified. A *healthy carrier* is one who harbours the pathogen but has never suffered from the disease caused by the pathogen, while a *convalescent carrier* is one who has recovered from the disease and continues to harbour the pathngen in his body. Depending on the duration of carriage, carriers are classified as *tempornry* and *chronic.* The temporary carrier state lasts less than six months, while chronic carriage may last for several years and sometimes even for the rest of one's life. The term *contact carrier* is applied to a person who acquires the pathogen from a patient while the term *paradoxical carrier* refers to a carrier who acquires the pathogen from another carrier.

Animals: Many pathogens are able to infect both man and animals. Animals may, therefore, act as sources of human infection. In some instances, the infection in animals may be asymptomatic. Such animals serve to maintain the parasite in nature and act as the *reservoir* of human infections. *They are, therefore, called reservoir hosts.* Infectious diseases transmitted from animals to man are called *zoonoses.* Zoonotic diseases may be bacterial (e.g., plague from rats), viral (e.g., rabies from dogs), protozoal (e.g., leishmaniasis from dogs), helminthic (e.g., hydatid disease frnm dogs) or fungal (e.g., zoophilic dermatophytes from cats and dogs).

Insects: Blood sucking insects may transmit pathogens to man. The diseases so caused are called *arthropod-borne diseases.* Insects such as mosquitoes, ticks, mites, flies, fleas and lice that transmit infections are called *vectors.* Transmission may be mechanical (e.g., transmission of dysentery or typhoid bacilli by the domestic fly). Such vectors are called *mechanical vectors.* In other instances, the pathogen multiplies in the body of the vector, often undergoing part of a developmental cycle in it. Such vectors are termed *biological vectors* (e.g., *Aedes aegypti* mosquito in yellow fever, Anopheles mosquito in malaria). Biological vectors transmit infection only after the pathogen has multiplied in them sufficiently or has undergone a developmental cycle. The interval of time required for the biological vector to become infective, beginning from the time of entry of pathogen into it, is termed the *extrinsic incubation period.*

Besides acting as vectors, some insects may also act as reservoir hosts (e.g., ticks in relapsing fever and spotted fever). Infection is maintained in such insects by transovarial or trans-stadial passage.

Soil nnd water: Some pathogens are able to survive in the soil for very long periods. Spores of tetanus bacilli may remain viable in soil for several decades and serve as the source of infection. Fungi (e.g., *Histoplasma capsulatum, Nocardia asteroides*) also survive in soil and cause human infection. Soil also serves as the source of parasitic infections such as roundworm and hookworm.

Water may act as the source of infection either due to contamination with pathogenic microorganisms (e.g., cholera vibrio, infective hepatitis virus) *or due to the presence of aquatic vectors* (e.g., cyclops in guinea worm infectinn).

Food: Contaminated food materials may act as sources of infection. Presence of pathogens in food may be due to external contamination (e.g., food poisoning by staphylococcus) or due to preexistent infecttion in meat or other animal prnducts (e.g., measly pork).

Methods of transmission of infection

Contact: Infection may be acquired by contact, which may be direct or indirect. Sexually trans-

mitted diseases such as syphilis and gonorrhoea illustrate spread by direct contact. The term *contagious disease* had been used for diseases transmitted by direct contact as distinct from the term *infectious disease* signifying all other modes of transmission. This distinction is now not generally employed. Indirect contact may be through the agency of *fomites*, which are inanimate objects, such as clothing, pencils or toys, which may be contaminated by a pathogen from one person and act as a vehicle for its transmission to another. Pencils shared by school children may act as fomites in the transmission of diphtheria, and face towels in trachoma.

Inhalation: Respiratory infections such as influenza and tuberculosis are transmitted by inhalation of the pathogen. Such microbes are shed into the environment by patients in secretions from the nose or throat during sneezing, speaking or coughing. Large drops of such secretions fall to the ground and dry there. Pathogens resistant to drying may remain viable in the dust and act as sources of infection. Small droplets, under 0.1 mm in diameter, evaporate immediately to become minute particles or *droplet nuclei* (usually 1–10μ in diameter) which remain suspended in air for long periods, acting as sources of infection.

Ingestion. Intestinal infections are generally acquired by the ingestion of food or drink contaminated with the pathogens. Infection transmitted by ingestion may be water-borne (cholera), food-borne (food poisoning) or hand-borne (dysentery). The importance of finger-borne transmission is being increasingly recognised, not only for pathogens entering through the mouth, but also those that enter through the nose and eyes.

Inoculation: Pathogens, in some instances, may be inoculated directly into the tissues of the host. Tetanus spores implanted in the depth of wounds, rabies virus deposited subcutaneously by dog bites and arboviruses injected by insect vectors are examples. Infection by inoculation may be iatrogenic when unsterile syringes and surgical equipment are employed. Hepatitis B and Human Immunodeficiency Virus may be transmitted through transfusion of infected blood, or the use of contaminated syringes and needles, particularly in addicts of injectable drugs.

Insects: Insects may act as mechanical or biological vectors of infectious diseases.

Congenital: Some pathogens are able to cross the placental barrier and infect the fetus *in utero*. This is known as vertical transmission. This may result in abortion, miscarriage or stillbirth. Live infants may be born with manifestations of the disease, as in congenital syphilis. Intrauterine infection with the rubella virus, especially in the first trimester of pregnancy, may interfere with organogenesis and lead to congenital malformation. Such infections are known as *teratogenic* infections.

Iatrogenic and laboratory infections: Infection may sometimes be transmitted during procedures such as injections, lumbar puncture and catheterisation, if meticulous care in asepsis is lacking. Modern methods of treatment such as exchange transfusion, dialysis, heart and transplant surgery increase the possibilities for iatrogenic infections. Laboratory personnel handling infectious material are at risk and special care should be taken to prevent laboratory infection.

The outcome of an infection will depend on the interaction between microbial factors which predispose to pathogenicity and host factors which contribute to resistance.

Factors predisposing to microbial pathogenicity

The terms 'pathogenicity' and 'virulence' refer to the ability of a microbe to produce disease or tissue injury, but it is convenient to make a fine distinction between them. 'Pathogenicity' is generally employed to refer to the ability of a microbial *species* to produce disease while the term 'viru-

lence' is applied to the same property in a *strain* of microorganism. Thus the species *M. tuberculosis* or the polio virus is referred to as pathogenic. The pathogenic species *M. tuberculosis* and the polio virus contain strains of varying degrees of virulence including those which are avirulent, such as the vaccine strains. The virulence of a strain is not constant and may undergo spontaneous or induced variation. Enhancement of virulence is known as *exaltation* and can be demonstrated experimentally by serial passage in susceptible hosts. Reduction of virulence is known as *attenuation* and can be achieved by passage through unfavourable hosts, repeated cultures in artificial media, growth under high temperature or in the presence of weak antiseptics, desiccation, or prolonged storage in culture.

Virulence is the sum total of several determinants, as detailed below.

Adhesion: The initial event in the pathogenesis of many infections is the attachment of the bacteria to body surfaces. This attachment is not a chance event, but a specific reaction between surface receptors on the epithelial cells and adhesive structures on the surface of bacteria. These adhesive structures are called *adhesins*. Adhesins may occur as organised structures, such as fimbriae or fibrillae and pili, or as colonisation factors. This specific adhesin may account for the tissue tropisms and host specificity exhibited by many pathogens. Adhesins serve as virulence factors. Loss of adhesins often renders the strain avirulent. Adhesins are antigenic. Specific immunisation with adhesins has been attempted as a method of prophylaxis in some infections, as for instance against *Escherichia coli* diarrhoea in calves and piglets, and gonorrhoea in humans.

Invasiveness: This refers to the ability of a pathogen to spread in the host tissues after establishing infection. Highly invasive pathogens characteris-

TABLE 9.1

Distinguishing features of exotoxins and endotoxins

	Exotoxins	*Endotoxins*
1	Proteins.	Protein-Polysaccharide-Lipid complexes
2	Heat labile.	Heat stable.
3	*Actively secreted by cells; diffuse* into surrounding medium.	*Form part of* cell wall; *do not* diffuse into surrounding medium.
4	Readily separable from cultures by physical means such as filtration.	Obtained only by cell lysis.
5	Action often enzymic.	No enzymatic action.
6	Specific pharmacological effect for each exotoxin	Effect nonspecific: action common to all endotoxins.
7	Specific tissue affinities	No specific tissue affinity.
8	Active in very minute doses.	Active only in very large doses
9	Highly antigenic.	Weakly antigenic.
10	Action specifically neutralised by antibody.	Neutralisation by antibody ineffective.
11	Can be toxoided.	Cannot be toxoided
12	Produced mainly by Gram positive bacteria, but also by some Gram negative bacteria.	Produced by Gram negative bacteria.

tically produce spreading or generalised lesions (e.g., streptococcal septicaemia following wound infection), while less invasive pathogens cause more localised lesions (e.g., staphylococcal abscess). Some pathogens though capable of causing serious or even fatal diseases lack invasiveness altogether (e.g., tetanus bacillus which remains confined to the site of entry and produces the disease by elaborating a potent toxin).

Toxigenicity: Bacteria produce two types of toxins, *exotoxins and endotoxins*. *Exotoxins* are heat labile proteins which are secreted by certain species of bacteria and diffuse readily into the surrounding medium. They are highly potent in minute amounts and constitute some of the most poisonous substances known. One mg of tetanus or botulinum toxin is sufficient to kill more than one million guinea pigs and it has been estimated that 3.0 kg of botulinum toxin can kill all the inhabitants of the world. Treatment of exotoxins with formaldehyde yields toxoids which are non-toxic but retain the ability to form antibodies (antitoxins). They exhibit specific tissue affinities and pharmacological activities, each toxin producing a typical effect which can be made out by characteristic clinical manifestations or autopsy appearances. Exotoxins are generally formed by Gram positive bacteria but may also be produced by some Gram negative organisms such as Shiga's *dysentery bacillus, cholera vibrio* and *enterotoxigenic E. coli*.

Endotoxins are heat stable polysaccharide-protein-lipid complexes which form an integral part of the cell wall of Gram negative bacteria. Their toxicity depends on the lipid component (lipid A). They are not secreted outside the bacterial cell and are released only by the disintegration of the cell wall. They cannot be toxoided. They are poor antigens and their toxicity is not completely neutralised by the homologous antibodies. They are active only in relatively large doses and lethal doses are of the order of 5–25 mg in the mouse. They do not exhibit specific pharmacological activities. All endotoxins, whether isolated from pathogenic or nonpathogenic bacteria, produce similar effects. *Administration of small quantities* of endotoxin in susceptible animals causes an elevation of body temperature appearing within 15 minutes and lasting for several hours. The pyrogenic effect of fluids used for intravenous administration is usually due to the presence in them of endotoxins from contaminant bacteria. Intravenous injections of large doses of endotoxin and massive Gram negative septicaemias cause the syndrome of endotoxic shock evidenced by fever, leucopaenia, thrombocytopaenia, profound fall of blood pressure, circulatory collapse and bloody diarrhoea leading to death (Tables 9.1, 9.2).

Communicability: The ability of a parasite to spread from one host to another is known as communicability. This property does not influence the production of disease in an individual host but determines the survival and distribution of a parasite in a community. A correlation need not exist between virulence and communicability. In fact, a highly virulent parasite may not exhibit a high degree of communicability due to its rapidly lethal effect on the host. In general, infections in

TABLE 9.2
Biological activities of endotoxins

1. Pyrogenicity; 2. Lethal action; 3. Depression of blood pressure; 4. Activation of complement; 5. Intravascular coagulation; 6. Leucopaenia; 7. Leucocytosis, Inhibition of glucose and glycogen synthesis in the liver, Stimulation of B lymphocytes; Macrophage inhibition; Interferon release; Induction of prostaglandin synthesis; *Clotting of limulus lysate* (lysate of amoebocytes from horse shoe crab, *Limulus polyphemus*, used as a test for detection of endotoxins).

which the pathogen is shed in secretions, as in respiratory or intestinal diseases, are highly communicable. *In some instances, as in hydrophobia,* human infection represents a dead end, there being an interruption in the spread of the pathogen to other hosts.

Development of epidemic and pandemic diseases requires that the strain of pathogen possesses high degrees of virulence and communicability.

Other bacterial products: Some bacterial products other than toxins, though devoid of intrinsic toxicity, may contribute to virulence by inhibiting the mechanisms of host resistence. Pathogenic staphylococci produce a thrombin-like enzyme coagulase which prevents phagocytosis by forming a fibrin barrier around the bacteria and walling off the lesion. Fibrinolysins promote the spread of infections by breaking down the fibrin barrier in tissues. Hyaluronidases split hyaluronic acid which is a component of intercellular connective tissue and thus facilitate the spread of infection along tissue spaces. Leucocidins damage polymorphonuclear leucocytes. Many pathogens produce haemolysins capable of destroying erythrocytes but their significance in pathogenicity is not clearly understood.

Bacterial appendages: Capsulated bacteria such as pneumococci, *K. pneumoniae* and *H. influenzae* are not readily phagocytosed. Some bacterial surface antigens such as the Vi antigen of *S. typhi*, K antigens of *E. coli* also help the bacteria to withstand phagocytosis and the lytic activity of complement.

Infecting dose: Successful infections require that an adequate number of bacteria should gain entry. The dosage may be estimated as the *minimum infecting dose* (MID) or *minimum lethal dose* (MLD) which are, respectively, the minimum number of bacteria required to produce clinical evidence of infection or death in a susceptible animal under standard conditions. As animals exhibit considerable individual variation in susceptibility, these doses are more correctly estimated as statistical expressions, ID 50 and LD 50, as the dose required to infect or kill 50 per cent of the animals tested under standard conditions.

Route of infection: Some bacteria, such as streptococci, can initiate infection whatever be the mode of entry. Others can survive and multiply only when introduced by the optimal routes. Cholera vibrios are infective orally but are unable to cause infection when introduced subcutaneously. This difference is probably related to modes by which different bacteria are able to initiate tissue damage and establish themselves. Bacteria also differ in their sites of election in the body after introduction into tissues. They also differ in the ability to produce damage of different organs in different species of animals. Tubercle bacilli injected into rabbits cause lesions mainly in the kidneys but infrequently in the liver and spleen, but in guinea pigs, the lesions are mainly in the liver and spleen, the kidneys being spared. The reasons for such selective multiplication in tissues, are largely obscure, though they may be related to the presence in tissues of substances that may selectively hinder or favour their multiplication.

Types of infectious diseases

Infectious diseases may be localised or generalised. Localised infections may be superficial or deepseated. Generalised infection involves the spread of the infecting agent from the site of entry by contiguity, through tissue spaces or channels, along lymphatics or through the bloodstream. MLS Circulation of bacteria in the blood is known as *bacteraemia*. Transient bacteraemia is a frequent event even in healthy individuals and may occur during chewing, brushing of teeth or straining at stools. The bacteria are immediately mopped up by phagocytic cells and are unable to initiate infection. Bacteraemia of greater severity and longer duration is seen during generalised infections as in typhoid fever. *Septicaemia* is the condition where bacteria circulate and multiply in the

blood, form toxic products and cause high, swinging type of fever. *Pyaemia* is a condition where pyogenic bacteria produce septicaemia with multiple abscesses in internal organs such as the spleen, liver and kidneys.

Depending on the spread of infectious disease in the community they may be classified into different types. *Endemic* diseases are those which are constantly present in a particular area. Typhoid fever is endemic in most parts of India. An *epidemic* disease is one that spreads rapidly, involving many persons in an area at the same time. Influenza causes annual winter epidemics in the cold countries. A *pandemic* is an epidemic that spreads through many areas of the world involving very large numbers of persons within a short period. Influenza, cholera, plague and enteroviral conjunctivitis are pandemic diseases. Epidemics vary in the rapidity of spread. Waterborne diseases such as cholera and hepatitis may cause explosive outbreaks while diseases which spread by person to person contact evolve more slowly. Such creeping or smouldering epidemics, as of cerebrospinal fever, are termed *prosodemic* disease.

Further Reading

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Bacteraemia : Circuln of bact. in blood

Septicaemia : Circuln & multiplicn of bact. in blood accomp by release of toxic products & fever

Pyaemia : Septicaemia c̄ multiple abscesses in Int. org eg. L, S, K

Endemic : disease c̄ is constantly pr. in an particular area.

Epidemic : disease c̄ spreads rapidly involving many persons in an area at a time.

Pandemic : disease c̄ spreads thru many areas of world & involve large no of people c̄ in a very short period.
eg. Influenza. Cholera plague.
AIDS

10 Immunity

The term 'immunity' refers to the resistance exhibited by the host towards injury caused by microorganisms and their products. Protection against infectious diseases is only one of the consequences of the immune response, which in its entirety is concerned with the reaction of the body against any foreign antigen.

Immunity against infectious diseases is of different types:

I INNATE IMMUNITY
- (a) Nonspecific
- (b) Specific

  } Species, Racial, Individual

II ACQUIRED IMMUNITY
- (a) Active — Natural; Artificial
- (b) Passive — Natural; Artificial

Innate or native immunity is the resistance to infections, which an individual possesses by virtue of his genetic and constitutional make-up. It does not depend on prior contact with microorganisms or immunisation. It may be nonspecific, when it indicates a degree of resistance to infections in general, or specific when resistance to a particular pathogen is concerned.

Innate immunity may be considered at the level of the species, race or individual. Species immunity refers to the total or relative refractoriness to a pathogen, shown by all members of a species. For instance, all human beings are totally insusceptible to plant pathogens and to many pathogens of animals such as rinderpest or distemper. This immunity is something a person obtains as a birthright, for the reason that he belongs to the human species. The mechanisms of species immunity are not clearly understood, but may be due to physiological and biochemical differences between the tissues of the different host species, which determine whether or not a pathogen can multiply in them. An early insight into the basis of species immunity was gained by Pasteur's experiments on anthrax in frogs, which are naturally resistant to the disease, but become susceptible when their body temperature is raised from 25°C to 35°C.

Within a species, different races may show differences in susceptibility to infections. This is known as racial immunity, the classic example of which is the high resistance of Algerian sheep to anthrax. Such racial differences are known to be genetic in origin, and by selection and inbreeding, it is possible to develop, at will, races that possess high degrees of resistance or susceptibility to various pathogens. It is difficult to demonstrate marked differences in immunity in human races, as controlled breeding is not possible in the human species. It has been reported that the Negroes in the U.S.A. are more susceptible than the whites to tuberculosis. But such comparisons are vitiated by external influences such as differences in socioeconomic levels. An interesting instance of genetic resistance to *Plasmodium falciparum* malaria is seen in some parts of Africa and the

Mediterranean coast. A hereditary abnormality of red cells (sickling), prevalent in the area, confers immunity to infection by the malarial parasite and may have evolved from the survival advantage conferred by it in a malarial environment.

The differences in innate immunity exhibited by different individuals in a race is known as individual immunity. The genetic basis of individual immunity is evident from studies on the incidence of infectious diseases in twins. It is well documented that homozygous twins exhibit similar degrees of resistance or susceptibility to lepromatous leprosy and tuberculosis. Such correlation is not seen in heterozygous twins.

Several factors influence the level of innate immunity in an individual:

Age: The two extremes of life carry a higher susceptibility to infectious diseases as compared to adults. The fetus *in utero* is normally protected from maternal infection by the placental barrier. But some pathogens cross this barrier causing overwhelming infections resulting in fetal death. Some, such as rubella and cytomegaloviruses and *Toxoplasma gondii*, lead to congenital malformations. The heightened susceptibility of the fetus to infection is related to the immaturity of its immune apparatus. Newborn animals are more susceptible to experimental infections than older ones. Coxsackie viruses cause fatal infection in suckling mice but not in the adults.

Increased susceptibility in the young may, in some instances, be due to hormonal influence. Tinea capitis caused by *Microsporum audouini* frequently undergoes spontaneous cure with the onset of puberty. The susceptibility of the vaginal epithelium in prepubertal girls to gonococcal infection is another instance of the effect of sex hormones on resistance.

Old persons are highly susceptible to infections due to the gradual waning of their immune responses.

Hormonal influences: Endocrine disorders such diabetes mellitus, hypothyroidism and adrenal dysfunction are associated with an enhanced susceptibility to infections. The high incidence of staphylococcal sepsis in diabetes may be related to the increased level of carbohydrates in tissues. Corticosteroids exert an important influence on the response to infection. They depress the host's resistance by their anti-inflammatory and antiphagocytic effects and by the suppression of antibody formation and hypersensitivity. They also have a beneficial effect in that they neutralise the harmful effect of bacterial products such as endotoxins. The elevated steroid level during pregnancy may have a relation to the heightened susceptibility of pregnant women to many infections. The reported effect of stress, in increasing susceptibility to infections, may in some measure be due to the release of steroids.

Nutrition. The interaction between malnutrition and immunity is complex, but in general, both humoral and cell mediated immune processes are reduced in malnutrition. Cell mediated immune responses such as the Mantoux test become negative in severe protein deficiency, as in Kwashiorkar. Because of its wide prevalence, malnutrition may well be the commonest form of immunodeficiency disease.

Paradoxically, there is some evidence that certain infections may not become clinically apparent in the severely ill nourished. Malarial infection in the famine stricken may not induce fever, but once their nutrition is improved, clinical malaria develops. It has also been reported that some viruses may not multiply in the tissues of severely malnourished individuals.

Mechanisms of Innate immunity

Epithelial surfaces. The intact skin and mucous membrane covering the body confer on it considerable protection against invasion by microorganisms. They provide much more than a mechanical barrier. The healthy skin possesses bactericidal activity to which the presence of high concentration of salt in the drying sweat, the

sebaceous secretions and the long chain fatty acids and soaps contribute. When cultures of typhoid bacilli placed on the healthy skin and on a glass surface are sampled at intervals, the bacteria on the skin are seen to be killed within minutes, while they survive for several hours on glass. The bactericidal activity of skin secretions is illustrated by the frequent mycotic and pyogenic infections seen in persons who immerse their hands in soapy water for long periods occupationally.

Though the skin frees itself readily of bacteria deposited on it (transients), its reactions are different to the bacterial flora normally resident on it. Resident flora are not easily removed even by washing and application of disinfectants.

The mucosa of the respiratory tract has several innate mechanisms of defence. The very architecture of the nose prevents entry of microorganisms to a large extent, the inhaled particles being arrested at or near the nasal orifices. Those that pass beyond are held by the mucus lining the epithelium, and are swept back to the pharynx where they tend to be swallowed or coughed out. The cough reflex is an important defence mechanism of the respiratory tract. The cilia on the respiratory epithelial cells propel particles upwards. Nasal and respiratory secretions contain mucopolysaccharides capable of neutralising influenza and some other viruses. Particles that manage to reach the pulmonary alveoli are ingested by the phagocytic cells present there.

The mouth is constantly bathed in saliva which has an inhibitory effect on many microorganisms. Particles deposited in the mouth are swallowed and subjected to the action of the digestive juices. The high acidity of the stomach destroys most microorganisms. The pH becomes progressively alkaline from the duodenum to the ileum. The duodenum and proximal jejunum contain few bacteria. The ileum contains a rich and varied flora and in the large intestine, the bulk of the contents is composed of bacteria. The normal bacterial flora exert a protective effect by preventing colonisation by pathogenic bacteria that may gain entry.

The intestinal mucosa is covered by a lace-like network of mucus. Particles get enmeshed in the mucus and form small masses which are propelled by peristalsis.

The conjunctiva is freed of foreign particles by the flushing action of lachrymal secretions. The eyes become susceptible to infection when lachrymal secretions are absent. Tears contain the antibacterial substance lysozyme, first described by Fleming (1922). This is a thermolabile substance producing lysis of some bacteria, and particularly of a nonpathogenic coccus, *Micrococcus lysodeikticus*. Lysozyme is present in tissue fluids and in nearly all secretions except in cerebrospinal fluid, sweat and urine. It acts by splitting certain polysaccharide components of the cell walls of susceptible bacteria. In the concentrations seen in tears and other secretions, lysozyme is active only against some nonpathogenic Gram positive bacteria. Its relevance in providing protection against pathogens is, therefore, uncertain. Recently it has been found to occur in phagocytic cells, in concentrations high enough to be lethal to many pathogens.

The flushing action of urine eliminates bacteria from the urethra. An antibacterial substance has been identified in the semen. The acidity of the adult vagina due to the fermentation of glycogen in the epithelial cells by the resident aciduric bacilli, renders it inhospitable for many pathogens.

Antibacterial substances in blood and tissues: The complement system possesses bactericidal activity and plays an important role in the destruction of pathogenic bacteria that invade the blood and tissues. Pillemer (1954) described *properdin*, a euglobulin present in normal serum, which along with complement and Mg^{++} causes lysis of Gram negative bacteria. It also inactivates some viruses. Properdin has a molecular weight of more than a million and constitutes 0.02 per cent of serum proteins. Properdin is now considered part of the antibacterial activity of the complement system.

Several substances possessing antibacterial property have been described in blood and tis-

sues. These include 1) beta lysin, a relatively thermostable substance active against anthrax and related bacilli, 2) basic polypeptides such as leukins extracted from leucocytes and plakins from platelets, and 3) acidic substances such as lactic acid found in muscle tissue and in the inflammatory zones. An antibacterial substance, spermine, has been isolated from the kidneys. While these substances possess antibacterial properties demonstable experimentally, their relevance in the natural situation is not clearly understood.

A method of defence against virus infections is the production of interferon by cells stimulated by live or killed viruses and certain other inducers. Interferon has been shown to be more important than specific antibodies in protection against and recovery from certain acute viral infections. Tissues and body secretions contain other antiviral substances such as inhibitors of viral haemagglutination and the virus inactivating agent (VIA). An antiviral substance has recently been identified in milk.

Cellular factor in innate immunity: Natural defence against invasion of blood and tissues by microorganisms and other foreign particles is mediated to a large extent by phagocytic cells which ingest and destroy them. Phagocytic cells, originally discovered by Metchnikoff (1833), were classified by him into microphages and macrophages. Microphages are polymorphonuclear leucocytes. Macrophages consist of the histiocytes which are the wandering amoeboid cells seen in tissues, the fixed reticuloendothelial cells and the monocytes of the blood. A major function of the reticuloendothelial system is the removal of foreign particles that enter the body. Phagocytic cells reach the sites of inflammation in large numbers attracted by chemotactic substances and ingest particulate materials. Capsulated bacteria, such as pneumococci, are not readily phagocytosed except in the presence of opsonins. They are more readily phagocytosed when trapped against a firm surface such as the alveolar wall, than when they are free in the tissue fluids. Bacteria are phagocytosed into a vacuole (phagosome), which fuses with the lysosomes found in the cell to form the phagolysosome. The bacteria are subjected to the action of the lytic enzymes in the phagolysosome and are destroyed. Some bacteria, such as brucella and lepra bacilli, resist intracellular digestion and may actively multiply inside the phagocytic cells. Phagocytosis in such instances may actually help to disseminate infections to different parts of the body. The importance of phagocytosis in protection against infection is evidenced by the enhanced susceptibility to infection seen either when the phagocytic cells are depleted, as in agranulocytosis, or when they are functionally deficient, as in chronic granulomatous disease.

Inflammation. Tissue injury or irritation initiated by the entry of pathogens or of other irritants leads to inflammation, which is an important, nonspecific mechanism of defence. The arterioles at the site constrict initially and then dilate leading to an increased blood flow. There is a slowing of blood flow and margination of the leucocytes. They tend to be arranged peripherally. The polymorphonuclear leucocytes escape into the tissues by diapedesis and accumulate in large numbers, attracted by the chemotactic substances released at the site of injury. Microorganisms are phagocytosed and destroyed. There is an outpouring of plasma which helps to dilute the toxic products present. A fibrin barrier is laid, serving to wall off the site of infection.

Fever: A rise of temperature following infection is a natural defence mechanism and helps not merely to accelerate physiological processes but may, in some cases, actually destroy the infecting pathogens. Therapeutic induction of fever was employed for destruction of *Treponema pallidum* in the tissues of syphilitic patients before penicillin became available. Fever stimulates the production of interferon and helps in recovery from virus infections.

Acquired immunity

The resistance that an individual acquires during life is known as acquired immunity, as distinct from the inborn innate immunity. Acquired immunity is of two types, active and passive. Active immunity is the resistance developed by an individual as a result of an antigenic stimulus. This involves the active functioning of the person's immune apparatus leading to the synthesis of antibodies and/or the production of immunologically active cells. Active immunity sets in only after a latent period which is required for the immunological machinery to be set in motion. During the development of active immunity, there is often a negative phase during which the level of measurable immunity may actually be lower than before the antigenic stimulus. This is due to the antigen combining with any preexisting antibody and lowering its level in circulation. Once developed, the active immunity is long lasting. Immunity is demonstrable for several years, in some instances. If an individual who has been actively immunised against an antigen experiences the same antigen subsequently, the immune response occurs more quickly and abundantly than during the first encounter. This is known as *secondary response.* Besides the development of humoral and cellular immunity, active immunity is associated with 'immunological memory'. This implies that the immune system is able to retain for long periods *the memory of a prior antigenic exposure and to* produce a secondary type of response when it meets with the same antigen again. Active immunisation is more effective and confers better protection than passive immunisation.

The resistance that is transmitted to a recipient in a 'ready-made' form is known as passive immunity. Here the recipient's immune system plays no active role. There is no antigenic stimulus; instead, preformed antibodies are administered. There is no latent period in passive immunity, protection being effective immediately following passive immunisation. There is no negative phase. The immunity is transient, lasting usually for days or weeks, only till the passively transmitted antibodies are metabolised and eliminated. No secondary type response occurs in passive immunity. In fact, passive immunity diminishes in effect with repetition. When a foreign antibody is administered a second time, it is eliminated more rapidly than initially. Following the first injection of an antibody, e.g., immune horse serum, the elimination is only by metabolic breakdown, but during subsequent injections of horse serum, its elimination is much quicker as it combines with antibodies to horse serum that would have been produced following its initial injection. This factor of 'immune elimination' limits the usefulness of repeated passive immunisation. Passive immunisation is less effective and provides an immunity inferior to that produced by active immunisation. The main advantage of passive immunisation is that it is immediate in action and, therefore, can be employed when 'instant' immunity is desired (Table 10.1).

Active immunity may be natural or artifical. Natural active immunity results from either a clinical or inapparent infection with a parasite. A person who has recovered from an attack of measles develops natural active immunity. The large majority of adults in the developing countries possess natural active immunity to poliomyelitis due to repeated inapparent infections with the polioviruses during childhood. Such immunity is usually longlasting, but the duration varies with the types of pathogen. The immunity *is lifelong following many viral diseases, such as* chicken pox or measles. In some viral diseases, such as influenza or the common cold, the immunity appears to be shortlived. Influenza can recur in an individual after a few months or a year, but this is not so much due to lack of the immunising capacity of the virus as to its ability to undergo antigenic variation so that immunity following the first infectinn is not effective against the second infection due to an antigenically novel virus. In common cold, the apparent lack of immunity is because the same clinical picture can be caused by infection with a large number of different viruses. The immunity following bacte-

9-11 V-V

TABLE 10.1
Comparison of active and passive immunity

	Active Immunity	Passive Immunity
1	Produced actively by the host's immune system	Received passively by the host. No participation by the host's immune system.
2	Induced by infection or by contact with immunogens (vaccines, allergens, etc.)	Conferred by introduction of readymade antibodies
3	Affords durable and effective protection.	Protection transient and less effective
4	Immunity effective only after a lag period (time required for generation of antibodies)	Immunity effective immediately
5	Immunological memory present, subsequent challenge more effective (booster effect)	No immunological memory; subsequent administration of antibody less effective due to 'immune elimination'
6	'Negative phase' may occur	No negative phase
7	Not applicable in immunodeficient hosts	Applicable in immunodeficient hosts

(*Note.* Adoptive Immunity, effected by transfer of Immunocompetent cells, shares the features of active and passive types of immunity It is discussed in Chapter 20)

rial infection is generally less permanent than that following viral infections. Some, such as typhoid fever, induce durable protection. In syphilis, a special type of immunity known as 'premunition' is seen. Here, the immunity to reinfection lasts only as long as the original infection remains active. Once the disease is cured, the patient becomes susceptible to the spirochaete again. In chancroid, another venereal disease, caused by *Haemophilus ducreyi*, there does not appear to be any effective immunity as the patient may develop lesions following reinfection even while the original infection is active.

Artificial active immunity is the resistance induced by vaccines Vaccines are preparations of live or killed microorganisms, or their products used for immunisation. Examples of vaccines are as follows:

I BACTERIAL VACCINES
a) Live — BCG for tuberculosis
b) Killed — TAB for enteric fever

II. VIRAL VACCINES
a) Live — Oral poliomyelitis
b) Killed — Salk vaccine for poliomyelitis

III BACTERIAL PRODUCTS
Toxoids for diphtheria and tetanus.

Live vaccines initiate an infection without causing any injury or disease. The immunity following live vaccine administration, therefore, parallels that following natural infection, though it may be of a lower order. The immunity lasts for several years, but booster doses may be necessary. Live vaccines may be administered orally (as with Sabin vaccine for poliomyelitis) or parenterally (as with measles vaccine). Killed vaccines are generally less immunogenic than live vaccines and protection lasts only for a short period They have, therefore, to be administered repeatedly, generally at least two doses being required for the production of immunity. The first is known as the primary dose and the subsequent doses as booster doses. Killed vaccines may be given orally

(e.g., Taboral vaccine for typhoid fever), but this route is generally not effective. Parenteral administration provides humoral antibody response, which may be improved by the addition of 'adjuvants' (e.g., Aluminium phosphate adjuvant vaccine for cholera).

Natural passive immunity is the resistance passively transferred from the mother to the baby. In human infants, maternal antibodies are transmitted predominantly through the placenta while in animals such as pigs, transfer of antibodies occurs mainly orally through the colostrum. Human colostrum which is also rich in IgA antibodies and resistant to intestinal digestion, confers protection to the neonate. The human fetus acquires some ability to synthesise antibodies (IgM) from about the twentieth week of life, but its immunological capacity is still inadequate at birth. It is only by about the age of three months that the infant acquires a satisfactory level of immunological independence. Maternal antibodies give passive protection against infectious diseases to the infant till then. Transport of antibodies across the placenta is an active process and, therefore, the concentration of antibody in the fetal blood may sometimes be higher than that seen in the mother. Protection so afforded will ordinarily be adequate against all the common infectious diseases in the locality. It is for this reason that most paediatric infections are commoner after the age of three months, than in younger infants. By active immunisation of mothers, during pregnancy, it is possible to improve the quality of passive immunity in the infants. Immunisation of pregnant women with tetanus toxoid is recommended for this purpose in communities in which neonatal tetanus is common.

Artificial passive immunity is the resistance passively transferred to a recipient by administration of antibodies. The agents used for this purpose are hyperimmune sera of animal or human origin, convalescent sera and pooled human gamma globulin. The oldest and the commonest method is to employ hyperimmune horse sera. These are prepared by active hyperimmunisation in horses using the appropriate antigen. For example, antitetanus serum (ATS) used for passive immunisation against tetanus is prepared by administering a series of doses of tetanus toxoid to horses, bleeding them and separating the serum. The antibodies in the serum are concentrated and purified by fractionation and enzyme treatment. The preparation is standardised to contain an adequate number of units of antitoxin per ml. ATS is administered parenterally — subcutaneously for prophylaxis and intravenously for treatment. ATS administration provides an immediate supply of the antitoxin in the recipient's circulation. As ATS is a foreign protein and liable to cause serious and even fatal hypersensitivity reactions, it should be administered only after prior testing for hypersensitivity. It is to eliminate the complication of hypersensitivity that human ATS is employed. This is prepared by hyperimmunisation of human volunteers with tetanus toxoid. An additional advantage with human ATS is that the protection lasts longer, there being no immune elimination of the human globulins.

Sera collected from patients convalescing from infectious diseases contain high levels of the specific antibody. Convalescent sera have been employed for passive immunisation against viral infections such as measles and rubella. Sera of healthy adults can be expected to contain antibodies against the infectious agents prevalent in the community. By pooling sera from a large number of adults in the community, the range of antibodies can be extended. The antibody fraction (gamma globulin) from such pooled sera is used for passive immunisation against common infectious diseases. The placenta provides a convenient source for the preparation of human gamma globulin. The use of human serum involves the risk of infectious hepatitis B and human immunodeficiency virus.

Passive immunisation is indicated for providing immediate and temporary protection in a non-immune host, faced with the threat of an infection, when there is insufficient time for active immunisation to take effect. It is also indicated

for treatment of infections. Passive immunisation may also be employed for the suppression of active immunity, when the latter may be injurious. An example is the use of antilymphocyte serum for the suppression of lymphocytes in situations such as transplant surgery when it is desired to suppress the immune responses towards the transplant. Another example is the use of Rh-immune globulin during delivery to prevent immune response to the Rhesus factor in Rh-negative women with Rh-positive babies.

Sometimes, a combination of active and passive methods of immunisation is employed simultaneously. This is known as 'combined immunisation'. Ideally, whenever passive immunisation is employed for providing immediate protection, combined immunisation is to be preferred, as in the protection of a nonimmune individual with a tetanus-prone wound. The method is to inject ATS in one arm and the first dose of tetanus toxoid in the other This is followed by the full course of phased tetanus toxoid injections. ATS provides the protection necessary till the active immunity is able to take effect.

A special type of immunisation is by injecting immunologically competent lymphocytes. This is known as 'adoptive immunity' and does not have general application. Instead of whole lymphocytes, an extract of immunologically competent lymphocytes, known as the 'transfer factor', can be used. This is being attempted in the therapy of certain types of disease (e.g., lepromatous leprosy).

Measurement of immunity

The truly valid measurement of immunity is to test the resistance of an individual to a challenge by the pathogen. But this is not applicable since the challenge itself alters the state of immunity. It is, therefore, not possible to measure accurately the level of immunity in an individual. Estimates of immunity are generally made using large numbers of individuals by statistical methods.

A simple method of testing immunity is to relate its level to some convenient indicator, such as demonstration of the specific antibody. But this is not always reliable as the immune response to a pathogen consists of the formation of antibodies to several antigens present in it. Such antibodies may be demonstrated by a variety of techniques such as agglutination, precipitation, complement fixation, haemagglutination inhibition and neutralisation. In the absence of exact information as to which of the antigens of the pathogen constitutes the 'protective antigen,' serological attempts to measure immunity are at best but approximations. In some instances, as in diphtheria where pathogenesis is due to a well defined antigen (the toxin), the level of immunity can be assayed by *in vitro* or *in vivo* (Schick test) methods. Where protection is associated with cell mediated immunity, skin tests for delayed hypersensitivity afford an indication of immunity.

Local Immunity

The concept of local immunity, proposed by Besredka (1919–24), has gained importance in the treatment of infections which are either localised or where it is operative in combating infection at the site of primary entry of the pathogen. In poliomyelitis, for instance, systemic immunity provided by active immunisation with the killed vaccine neutralises the virus when it enters the bloodstream. But it does not prevent multiplication of the virus at the site of entry (the gut mucosa) and its faecal shedding. This is achieved by the local intestinal immunity acquired as a result of either natural infection or immunisation with the live oral vaccine. In influenza, immunisation with the killed vaccine elicits a humoral antibody response. But the antibody titre in respiratory secretions is often not high enough to prevent infection. Natural infection or the live virus vaccine administered intranasally provides local immunity. A special class of immunoglobulins (IgA) forms the major component of local immunity.

One type of IgA antibody called *secretory* IgA

is produced locally by plasma cells present on mucosal surfaces or in secretory glands. There appears to be a selective transport of such antibodies between the various mucosal surfaces and secretory glands. Thus, following intestinal exposure to an antigen, the specific IgA antibody and plasma cells forming such antibody can be demonstrated in breast milk. This indicates the existence of a common *mucosal* or *secretory immune system*. Besides providing local defence against microorganisms, the mucosal immune system may also be involved in handling various antigens that may come into contact with mucosal surfaces from the external environment or through food.

Herd immunity

This refers to the overall level of immunity in a community and is relevant in the control of epidemic diseases. When a large proportion of individuals in a community (herd) are immune to a pathogen, the herd immunity to the pathogens is satisfactory. When herd immunity is low, epidemics are likely to occur on the introduction of a suitable pathogen, due to the presence of large numbers of susceptible individuals in the community. Eradication of communicable diseases depends on the development of a high level of herd immunity rather than in the development of high level of immunity in individuals.

Further Reading

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① Protein } Complete Ag
② Reaction } → (Hapten + carrier)
③ Delayed hypersensitivity - proantigen

11 Antigens

① picryl chloride
② $(NO_2)_3(Cl)$ ⬡

An antigen has been defined as any substance which, when introduced parenterally into the body, stimulates the production of an antibody with which it reacts specifically and in an observable manner. This traditional description of an antigen is no longer comprehensive enough in the light of current concepts about the immune response. Some antigens may not induce antibodies but may lead to cell mediated immunity or immunological tolerance

The two attributes of antigenicity are: 1) induction of an immune response (immunogenicity), and 2) specific reaction with antibodies or sensitised cells (immunological reactivity). Based upon the ability of antigens to carry out these two functions, they may be classified into different types. A *complete antigen* is able to induce antibody formation and produce a specific and observable reaction with the antibody so produced *Haptens* are substances which are incapable of inducing antibody formation by themselves, but can react specifically with antibodies. (The term hapten is derived from Greek *haptein* = to fasten). Haptens become immunogenic (capable of inducing antibodies) on combining with a larger molecule *carrier*. Haptens may be complex or simple; while *complex haptens* can precipitate with specific antibodies, *simple haptens* are nonprecipitating. They can inhibit precipitation of specific antibodies by the corresponding antigen or complex hapten Complex and simple haptens have been described as polyvalent and univalent, respectively, since it is assumed that precipitation requires the antigen to have two or more antibody combining sites

The term *proantigen* has been used to refer to low molecular weight substances, such as picryl chloride and dinitrochlorobenzene, which do not induce antibody formation but cause delayed hypersensitivity (cell mediated immunity) when applied on the skin. They appear to act by combining with autologous proteins. *Cryptantigens* are antigenic determinants that may not be available during antigen-antibody reactions because they are not terminal The term *pseudo-cryptantigen* has been used to refer to antigens which are not available for serological reaction due to steric hindrance as they are hidden or buried beneath the surface The smallest unit of antigenicity is known as the *antigenic determinant* (or *epitope*). This is represented by a small area on the antigen possessing a specific chemical structure and steric (spatial) configuration which determine the specificity of the antibody induced and of the serological reaction Antigens possess a number of determinant groups or combining sites. The determinant groups on protein antigens consist of penta- or hexapeptides and on polysaccharides, of hexasaccharides The determinant group has been estimated to have a size of around 25–34 Å and a molecular weight of about 400–1000. Large antigens carry many different types of antigenic determinants, presenting an antigenic mosaic. Each type of determinant induces the formation of a specific antibody

Determinants of antigenicity

A number of properties have been identified

which make a substance antigenic, but the exact basis of antigenicity is still not clear.

Size: Antigenicity bears a relation to molecular size. Very large molecules, such as haemocyanins (M.W. 6.75 million), are highly antigenic and particles with low molecular weight (less than 10,000) are nonantigenic or feebly so. Low molecular weight substances may be rendered antigenic by adsorbing them on large inert particles such as bentonite or kaolin.

Chemical nature: Most naturally occurring antigens are proteins and polysaccharides. Lipids and nucleic acids are less antigenic. Their antigenicity is enhanced by combination with proteins. Not all proteins are, however, antigenic. A well known exception is gelatin. It has been suggested that the presence of an aromatic radical is essential for antigenicity and the nonantigenicity of gelatin has been ascribed to the absence of aromatic aminoacids, such as tyrosine, in it.

D-AA
L-AA

Susceptibility to tissue enzymes: Only substances which are metabolised and are susceptible to the action of tissue enzymes behave as antigens. Antigens introduced into the body are degraded by the host into fragments of appropriate size containing the antigenic determinants. Phagocytosis and intracellular enzymes appear to play an essential role in breaking down antigens into immunogenic fragments. Substances insusceptible to tissue enzymes such as polystyrene latex are not antigenic. Synthetic polypeptides composed of D-aminoacids which are not metabolised in the body are not antigenic, while polypeptides consisting of L-aminoacids are antigenic.

Foreignness: Only antigens which are 'foreign' to the individual (non-self) induce an immune response. The animal body contains numerous antigens which induce an immune response when introduced into another individual or species. An individual does not normally mount an immune response against his own normal constituent antigens. This was first recognised by Ehrlich who proposed the concept of 'horror autotoxicus'. Tolerance of self-antigens is conditioned by contact with them during the development of the immune apparatus. Breakdown of this homeostatic mechanism results in autoimmunisation and autoimmune disease.

In general, the antigenicity of a substance is related to the degree of its foreignness. Antigens from other individuals of the same species are less antigenic than those from other species. Antigens from related species are less antigenic than those from distant species.

Antigenic specificity: The basis of antigenic specificity is stereochemical, as was first demonstrated by Obermayer and Pick and confirmed by subsequent work by Landsteiner. Using haptens such as atoxyl coupled with protein, it was shown that antigenic specificity is determined by single chemical groupings and even by a single acid radical. The importance of the position of the antigenic determinant group in the antigen molecule was evidenced by the differences in specificity in compounds with the group attached at *ortho*, *meta* or *para* positions. The influence of spatial configuration of the determinant group was shown by differences in antigenic specificity of *dextro*, *laevo* and *meso* isomers of substances such as tartaric acid.

Antigenic specificity is not absolute. Cross reactions can occur between antigens which bear stereochemical similarities. In some instances, apparent cross reactions may actually be due to the sharing of identical antigenic determinants by different antigens.

The specificity of natural tissue antigens of animals may be considered under different categories as *species*, *iso-*, *auto-*, and *organ* specificities.

SSA

Species specificity: Tissues of all individuals in a species contain species specific antigens. There exists some degree of cross reaction between antigens from related species. This immunological relationship parallels phylogenetic relation-

ships. Such immunological relationships between species specific antigens have been of assistance in tracing evolutionary relationships. Species specific antigens also have forensic applications in the identification of the species of blood and of seminal stains. Phylogenetic relationships are reflected in the extent of cross reaction between antigens from different species that cause hypersensitivity. An individual sensitised to horse serum will react with serum from other equines but may not do so with bovine serum.

Isospecificity: Isoantigens are antigens found in some but not all members of a species. A species may be grouped depending on the presence of different isoantigens in its members. The best examples of isoantigens are the human erythrocyte antigens based on which individuals can be classified into different blood groups. These are genetically determined. Besides being of clinical importance in blood transfusion and in isoimmunisation during pregnancy, they may provide valuable evidence in disputed paternity and find application in anthropology.

Histocompatibility antigens are those cellular determinants specific for each individual of a species that are recognised by genetically different individuals of the same species when attempts are made to transfer or transplant cellular material from one individual to another. These antigens are associated with the plasma membranes of tissue cells. Several human and animal histocompatibility antigens have been identified. The major histocompatibility antigens determining homograft rejection in man are antigens of the HL-A (Human leucocyte antigen) system. The major histocompatibility system is designated H-2 in mice, AgB in rats and B in fowls.

Autospecificity: Autologous or self-antigens are ordinarily nonantigenic, but there are exceptions. Sequestrated antigens that are not normally found free in circulation or tissue fluids (such as lens protein normally confined within its capsule) are not recognised as self-antigens. Similarly, antigens that are absent during the embryonic life and develop later (such as the sperm) are also not recognised as self-antigens. When these antigens are released into the tissues, as for instance following injury to the lens or damage to the testis, antibodies are produced against them. This is one mechanism in the pathogenesis of autoimmune diseases. The antigenic specificity of self-antigens may undergo minor modification as a result of infections or irradiation and may thus become immunogenic. The immune response so induced may lead to autoimmune disease. Autoimmune processes may also be set in motion by the failure of homeostatic control of immune function.

Organ specificity: Some organs, such as the brain, kidney and lens protein of different species, share the same antigen. Such antigens characteristic for an organ or tissue, found in different species, are called organ specific antigens. The neuroparalytic complications following antirabic vaccination are a consequence of brain specific antigens shared by sheep and man. Antirabic vaccine contains large amounts of partially denatured sheep brain tissue. In man they induce immunological response causing damage to the recipient nervous tissue.

Heterogenetic (heterophile) specificity: The same or closely related antigens may sometimes occur in different biological species, classes and kingdoms. These are known as heterogenetic or heterophile antigens. The best known example of such heterophile antigen is the Forssman antigen which is a lipid carbohydrate complex widely distributed in man, animals, birds, plants and bacteria. Other heterophile antigens are responsible for some diagnostic serological reactions in which antigens unrelated to aetiological agents are employed (heterophile reaction). The Weil-Felix reaction in typhus fever, Paul Bunnel test in infectious mononucleosis and cold agglutinin test in primary atypical pneumonia are examples.

Further Reading

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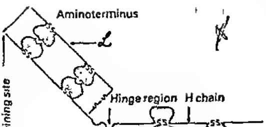


Fig 12 2a The four-peptide chain structure of the IgG molecule composed of two identical heavy (H) and two identical light (L) chains linked by interchains disulphide bonds. Loops formed by intrachain disulphide bonds are domains (shown stippled) Each chain has one domain in the variable region (VH and VL). Each light chain has one domain the constant region (CL) while each heavy chain has three domains in the constant region (CH1, 2 and 3) Between CH1 and CH2 is the hinge region.

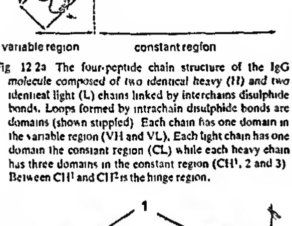


Fig. 12 2b Secretory IgA 1 heavy chain 2 light chain 3 J chain 4. secretory component 5 disulphide bond

lambda chains, but never both together. *Kappa* and *lambda* are named after the investigators Korngold and Lapari who originally described them. The *kappa* and *lambda* chains occur in a ratio of about 2:1 in human sera.

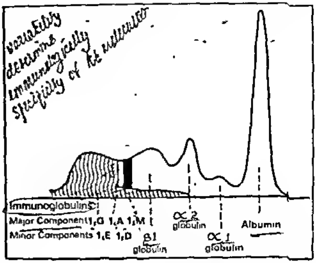
The antigen-combining site of the molecule is at its aminoterminus. It is composed of both L and H chains. Studies on the primary structure of L and H chains show that they consist of two portions each. Of the 214 aminoacid residues that make up the L chain, about 107 that constitute the carboxyterminal half occur only in a constant sequence. This part of the chain is, therefore, called the 'constant region'. Only two sequence patterns are seen in the constant region — those determining the *kappa* and *lambda* specificities. On the other hand, the aminoacid sequence in the aminoterminal half of the chain is highly variable, the variability determining the immunological specificity of the antibody molecule. It is, therefore, called the 'variable region'. The H chain also has 'constant' and 'variable' regions. But, while in L chains the two regions are of equal length, in H chains the variable region constitutes approximately only a fifth of the chain and is located at its aminoterminus. The infinite range of antibody specificity of immunoglobulins depends on the variability of the aminoacid sequences at the 'variable regions' of the H and L chains which form the antigen-combining sites.

The aminoacid sequences of the variable regions of L and H chains are not uniformly variable along their length, but consist of relatively invariable and some highly variable zones. The highly variable zones numbering three in L and four in H chains are known as *hypervariable regions* (or hot spots) and are involved with the formation of the antigen binding sites.

The *Fc* fragment is composed of the carboxyterminal portion of the H chains. It does not possess antigen-combining activity, but determines the biological properties of the immunoglobulin molecule such as complement fixation, placental transfer, skin fixation and catabolic rate. The portion of H chain present in the *Fab* fragment is called *Fd* piece. The H chain carries a carbohydrate moiety which is distinct for each class of immunoglobulins.

Each immunoglobulin peptide chain has internal disulphide links in addition to interchain disulphide bonds which bridge H and L chains.

These intrachain disulphide bonds form loops in the peptide chain and each of the loops is compactly folded to form a globular *domain*, and each domain has its separate function. The variable region domains, V_L and V_H, are responsible for the formation of a specific antigen binding site. The CH2 region in IgG binds C_{1q} in the classical complement sequence and CH3 domain mediates adherence to the monocyte surface. The area of the H chain in the C region between the first and second C region domains (CH1 and CH2) is the *hinge region*. It is more flexible and is more exposed to enzymes and chemicals. Papain acts here to produce two *Fab* and one *Fc*, fragments.



SM Fig. 12 3 Electrophoretic pattern of human serum showing the main components

Immunoglobulin classes

Human sera contain IgG, IgA, IgM, IgD and IgE in order of descending concentrations. Table 12.1 shows their characteristics.

IgG: This is the major serum immunoglobulin, constituting about 80 per cent of the total. It has a molecular weight of 150,000 (7S). IgG may occasionally exist in a polymerised form. It is distributed approximately equally between the intravascular and extravascular compartments. It contains less carbohydrates than other immunoglobulins. It has a half life of approximately 23 days. The catabolism of IgG is unique in that it varies with its serum concentration. When its level is raised, as in chronic malaria, kala azar or myeloma, the IgG synthesised against a particular antigen will be catabolised rapidly and may result in the particular antibody deficiency. Conversely, in hypogammaglobulinaemia, the IgG given for treatment will be catabolised only slowly. The normal serum concentration of IgG is about 8–16 mg per ml. MCQ

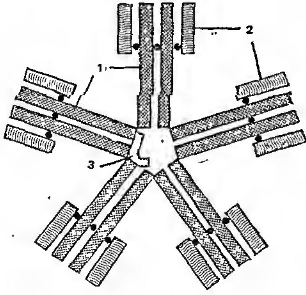


Fig 12 2c IgM molecule. 1. heavy chain 2. light. 3 J. chain

MCQ IgG is the only maternal immunoglobulin that is normally transported across the placenta and provides natural passive immunity in the new born. It is not synthesised by the fetus in any significant amount. IgG binds to microorganisms and enhances their phagocytosis. Extracellular killing of target cells coated with IgG antibody is mediated through recognition of the surface *Fc* fragment by K cells bearing the appropriate receptors. Interaction of IgG complexes with platelet *Fc* receptors probably leads to aggregation and vasoactive amine release. IgG alone, among the human immunoglobulins, has the property of fixing itself to guinea pig skin but the significance of such a property is not known. IgG participates in most immunological reactions

such as complement fixation, precipitation, and neutralisation of toxins and viruses. It may be considered as a general purpose antibody, protective against those infectious agents which are active in the blood and tissues. Passively administered IgG suppresses the homologous antibody synthesis by a feedback process. This property is utilised in the isoimmunisation of women by the administration of anti-Rh(D) IgG during delivery. With most antigens, IgG is a late antibody and makes its appearance after the initial immune response which is IgM in nature.

Four subclasses of IgG have been recognised (IgG1, IgG2, IgG3, IgG4), each possessing a distinct type of gamma chain, identifiable with specific antisera. The four IgG subclasses are distributed in human serum in the approximate *proportions of 65 per cent, 23 per cent, 8 per cent* and 4 per cent, respectively.

IgA: IgA is the second most abundant class, constituting about 10–13 per cent of serum immuno*globulins. The normal serum level is* 0.6–4.2 mg per ml. It has a half life of 6–8 days. It is the major immunoglobulin in colostrum, saliva and tears. It occurs in two forms. Serum IgA is principally a 7S molecule (M.W. about 160,000). IgA found in secretions contains an additional structural unit called the transport (T) or secretory (S) piece. The T piece is synthesised not in the lymphoid cells, but in the epithelial cells of glands, intestines and the respiratory tract, and is attached to the IgA molecules during transport across the cells. The T piece links together two IgA molecules at their Fc portion, producing 11S dimers. Another polypeptide chain, the J chain, has been demonstrated in polymeric forms of IgA and IgM. The J chain (joining chain) is synthesised by the lymphoid cells.

IgA found in secretions is largely synthesised locally by plamsa cells in the tissues; but a small amount may be derived from serum. Dimeric IgA antibody is selectively and rapidly transported across the hepatic parenchymal cells

TABLE 12.1
Some properties of immunoglobulin classes

	IgG	IgA*	IgM	IgD	IgE
Sedimentation coefficient (S)	7	7	19	7	8
Molecular weight	150,000	160,000	900,000	180,000	190,000
Serum concentration (mg/ml)	12	2	1.2	0.03	0.00004
Half life (days)	23	6	5	2·8	1·5
Daily production (mg/kg)	34	24	3.3	0.4	0.0023
Intravascular distribution (per cent)	45	42	80	75	50
Carbohydrate (per cent)	3	8	12	13	12
Complement fixation					
Classical	++	–	+++	–	–
Alternative	–	+	–	–	–
Placental transport	+	–	–	–	–
Present in milk	+	+	–	–	–
Selective secretion by seromucus glands	–	+	–	–	–
Heat stability (56°C)	+	+	+	+	–

* IgA may occur in 7S, 9S and 11S forms.

into the bile. IgA is selectively concentrated in secretions and on mucus surfaces, forming an 'antibody paste' and is believed to play an important role in local immunity against respiratory and intestinal pathogens. Secretory IgA is relatively resistant to digestive enzymes and reducing agents. IgA antibodies may function by inhibiting the adherence of microorganisms to the surface of mucosal cells by covering the organisms and thereby preventing their entry into body tissues. IgA does not fix complement but can activate the alternate complement pathway. It promotes phagocytosis and intracellular killing of microorganisms.

Two IgA subclasses have been described, IgA_1 and IgA_2. IgA_2 lacks interchain disulphide bonds between heavy and light chains. Though IgA_2 is a minor component of serum IgA, it is the dominant form in the secretions.

IgM: IgM constitutes 5–8 per cent of serum immunoglobulins, with a normal level of 0.5–2 mg per ml. It has a half life of about five days. It is a heavy molecule (19S; M.W. 900,000 to 1,000,000, hence called "the millionaire molecule"). IgM molecules are polymers of five, fourpeptide subunits, each bearing an extra CH domain. As with IgA, polymerisation of the subunits depends upon the presence of the J chain. Though the theoretical valency is ten, this is observed only with small haptens. With larger antigens, the effective valency falls to five, probably due to steric hindrance. Most of IgM (80 per cent) is intravascular in distribution. Phylogenetically, IgM is the oldest immunoglobulin class. It is also the earliest immunoglobulin to be synthesised by the fetus, beginning by about 20 weeks of age. As it is not transported across the placenta, the presence of IgM in the fetus or newborn indicates intrauterine infection and its detection is useful in the diagnosis of congenital syphilis, rubella and toxoplasmosis. IgM antibodies are relatively shortlived, disappearing earlier than IgG. Hence, their demonstration in serum indicates recent infection. Treatment of serum with 0.12M 2-mercaptoethanol selectively destroys IgM without affecting IgG antibodies. This provides a simple method for the differential estimation of IgG and IgM antibodies.

The isohaemagglutinins (anti-A, anti-B) and many other natural antibodies to microorganisms are usually IgM; antibodies to typhoid 'O' antigen (endotoxin) and WR antibodies in syphilis also tend to be found in this class.

Preliminary studies, by peptide mapping and complement fixing activity, indicate the existence of at least two subclasses of IgM, IgM_1 and IgM_2, characterised by μ_1 and μ_2 H chains, respectively. The unique structural features of IgM appear to be particularly suited for the biological role of providing protection against microorganisms and other large antigens that have repeating antigenic determinants on their surface. A single molecule of IgM can bring about immune haemolysis, whereas 1000 IgG molecules are required for the same effect. IgM is also 500–1000 times more effective than IgG in opsonisation, a 100 times more effective in bactericidal action and about 20 times in bacterial agglutination. But in neutralisation of toxins and viruses, it is less active than IgG. Being largely confined to the intravascular space, IgM is believed to be responsible for protection against blood invasion by microorganisms. IgM deficiency is often associated with septicaemias.

IgD: IgD resembles IgG structurally. It is present in a concentration of about 3 mg per 100 ml of serum and is mostly intravascular. It has a half life of about three days. IgD and IgM occur on the surface of unstimulated B lymphocytes and serve as recognition receptors for antigens. Combination of cell membrane-bound IgD or IgM with the corresponding antigen leads to specific stimulation of the B cell — either activation and cloning to produce antibody, or suppression. Two subclasses IgD_1 and IgD_2 have been described.

IgE: This immunoglobulin was discovered in 1966 by Ishizaka during the investigation of atopic reagin antibodies. It is an 8S molecule (M.W. about 190,000), with a half life of about

two days. It resembles IgG structurally. It exhibits unique properties such as heat lability (inactivated at 56°C in one hour) and affinity for the surface of tissue cells (particularly mast cells) of the same species (homocytotropism). It mediates the Prausnitz-Kustner reaction. It is susceptible to mercaptoethanol. It does not pass the placental barrier or fix complement. It is mostly extravascular in distribution. Normal serum contains only traces (a few nanograms per ml), but greatly elevated levels are seen in atopic (Type I allergic) conditions such as asthma, hay fever and eczema. Children living in insanitary conditions, with a high load of intestinal parasites, have high serum levels of IgE.

IgE is chiefly produced in the linings of the respiratory and intestinal tracts. Deficiency of IgE has been inconstantly associated with IgA deficiency in individuals with impaired immunity who present undue susceptibility to infection.

IgE is responsible for the anaphylactic type of hypersensitivity. But apart from this undesirable effect, no beneficial function of the antibody has so far been identified. It has been suggested that it may play some as yet unidentified role in the defence against intestinal parasitic infection.

From available information on the functional significance of the immunoglobulin classes, it appears that IgG protects the body fluids, IgA the body surfaces and IgM the bloodstream, while IgE mediates reaginic hypersensitivity. The role of IgD is unknown.

Abnormal immunoglobulins

Apart from antibodies, other structurally similar proteins are seen in serum in many pathological processes, and even sometimes in healthy persons. The earliest description of an abnormal immunoglobulin was the discovery by Bence-Jones (1847) of the protein that bears his name. Bence-Jones protein is found typically in multiple myeloma. It can be identified in urine by its characteristic property of coagulating when heated to 60°C, but redissolving at 80°C. Bence-Jones proteins are the light chains of immunoglobulins and so may occur as the *kappa* or *lambda* forms. But in any one patient, the chain is either *kappa* or *lambda* only, and never both, being uniform in all other respects also. This is because myeloma is a plasma cell dyscrasia in which there is unchecked proliferation of one clone of plasma cells, resulting in the excessive production of the particular immunoglobulin synthesised by the clone. Such immunoglobulins are, therefore, called monoclonal.

Multiple myeloma may affect plasma cells synthesising IgG, IgA, IgD or IgE. Similar involvement of IgM-producing cells is known as Waldenstrom's macroglobulinaemia. In this condition, there occurs excessive production of the respective myeloma proteins (M proteins) and of their light chains (Bence-Jones proteins). A different disorder is found in 'heavy chain disease', which is a lymphoid neoplasia characterised by the over-production of the *Fc* parts of immunoglobulin heavy chains.

Cryoglobulinaemia is a condition in which there is the formation of a gel or a precipitate on cooling the serum, which redissolves on warming. It may not always be associated with disease, but is often found in myelomas, macroglobulinaemias and autoimmune conditions such as systemic lupus erythematosus. Most cryoglobulins consists of either IgG, IgM or their mixed precipitates.

Because of the monoclonal nature of Bence-Jones and other M proteins, they have been valuable models for the understanding of immunoglobulin structure and function.

Immunoglobulin specificities

The immunoglobulin specificity of the greatest biological importance is immunological specificity. As already indicated, this is determined by the aminoacid sequences on the variable regions of the H and L chains.

Immunoglobulins exhibit other genetically determined specificities based on their antigenic structure. The antigenic specificities which distinguish between the different classes and sub-

classes of immunoglobulins present in all normal individuals of a given species are termed *isotypic* specificities. Antigenic specificities which distinguish immunoglobulins of the same class, between different groups of individuals in the same species, are called *allotypic* specificities.

Antigenic specificity exclusive to each immunoglobulin molecule is called the *idiotype*. Idiotypic determinants are located at the hypervariable regions of the molecule, which constitute the antigen-combining sites. Auto-anti-idiotypic antibodies occur naturally to immunoglobulin molecules produced in the body and form the basis of Jerne's network hypothesis of immunoregulation.

Immunoglobulin allotypes have been studied in detail in the rabbit and guinea pig by using type specific immune sera. Such deliberate immunisation is not possible in man, but anti-allotype specific antibodies may be produced following blood tranfusion or passage of maternal IgG into the fetus. Anti-allotype antibodies are also found in sera carrying the 'rheumatoid arthritis factor'.

Two allotypic systems are known in man — the *Gm system* (for *gamma* marker) and the *In V system* (abbreviation of patient's name) The Gm is associated with the *Fc* portion of the IgG heavy chain. More than 25 Gm types have been identified so far. The InV system is associated with the *kappa* light chain. Three InV allotypes have been identified.

Genetic markers associated with IgA are called 'Am'. To date, in the human system no allotypic markers have been found for λ light chain or μ, δ or ϵ heavy chains.

Further Reading

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13 Antigen-Antibody Reaction

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Antigens and antibodies, by definition, combine with each other specifically and in an observable manner. The reactions between antigens and antibodies serve several purposes. In the body, they form the basis of antibody mediated immunity in infectious diseases, or of tissue injury in some types of hypersensitivity and autoimmune diseases. In the laboratory, they help in the diagnosis of infections, in epidemiological surveys, in the identification of infectious agents and of noninfectious antigens such as enzymes. In general, these reactions can be used for the detection and quantitation of either antigens or antibodies. Antigen-antibody reactions *in vitro* are known as serological reactions.

The reactions between antigens and antibodies occur in three stages. The primary stage is the initial interaction between the two, without any visible effects. This reaction is rapid, occurs even at low temperatures and obeys the general laws of physical chemistry and thermodynamics. The reaction is reversible, the combination between antigen and antibody molecules being effected by the weaker intermolecular forces such as Van der Waal's forces, ionic bonds and hydrogen bonding, rather than by the firmer covalent bonding. The primary reaction can be detected by estimating free and bound antigen or antibody separately in the reaction mixture by a number of physical and chemical methods, including the use of markers such as radioactive isotopes, fluorescent dyes or ferritin.

In most instances, but not all, the primary stage is followed by the secondary stage leading to demonstrable events such as precipitation, agglutination, lysis of cells, killing of live antigens, neutralisation of motile organisms and enhancement of phagocytosis. When such reactions were discovered one by one, it was believed that a different type of antibody was responsible for each type of reaction and the antibodies came to be designated by the reactions they were thought to produce. Thus, the antibody causing agglutination was called agglutinin, that causing precipitation precipitin, etc. and the corresponding antigen, agglutinogen, precipitinogen, etc. By the 1920s, this view was replaced by Zinsser's unitarian hypothesis which held that an antigen gave rise to only one antibody, which was capable of producing all the different reactions depending on the nature of the antigen and the conditions of the reaction. Both these extreme views are fallacious. While it is true that a single antibody can cause precipitation, agglutination and most of the other serological reactions, it is also true that an antigen can stimulate the production of different classes of immunoglobulins which differ in their reaction capacities as well as in other properties (Table 13.1).

Some antigen-antibody reactions occurring *in vivo* initiate chain reactions that lead to neutralisation or destruction of injurious antigens, or to tissue damage. These are the tertiary reactions and include humoral immunity against infectious disease as well as clinical allergy and other immunological diseases.

General features of antigen-antibody reactions

Antigen-antibody reactions have the following general characteristics:

TABLE 13.1
Comparative efficiency of the immunoglobulin classes in different serological reactions

Reaction	IgG	IgM	IgA
Precipitation	Strong	Weak	Variable
Agglutination	Weak	Strong	Moderate
Complement fixation	Strong	Weak	Negative
Lysis	Weak	Strong	Negative

1. The reaction is specific, an antigen combining only with its homologous antibody and vice versa. The specificity, however, is not absolute and 'cross reactions' may occur due to antigenic similarity or relatedness.
2. Entire molecules react and not fragments. When an antigenic determinant present in a large molecule or on a 'carrier' particle reacts with its antibody, whole molecules or particles are agglutinated.
3. *There is no denaturation* of the antigen or the antibody during the reaction.
4. The combination occurs at the surface. Therefore, it is the surface antigens that are immunologically relevant. Antibodies to the surface antigens of infectious agents are generally protective.
5. The combination is firm, but reversible. The firmness of the union is influenced by the affinity and avidity of the reaction. Affinity refers to the intensity of attraction between antigen and antibody molecules. Avidity is the strength of the bond after the formation of antigen-antibody complexes.
6. Both antigens and antibodies participate in the formation of agglutinates or precipitates.
7. Antigens and antibodies can combine in varying proportions: unlike chemicals with fixed valencies. Both antigens and antibodies are multivalent. Antibodies are generally bivalent, though IgM molecules may have five or ten combining sites. Antigens may have valencies upto hundreds.

Measurement of antigen and antibody

Many methods are available for the measurement of antigens and antibodies participating in the primary, secondary or tertiary reactions. Measurement may be in terms of mass (e.g., mg Nitrogen) or more commonly as units or titre. The antibody titre of a serum is the highest dilution of the serum which gives an observable reaction with the antigen in the particular test. The titre of a serum is influenced by the nature and quantity of the antigen and the type and conditions of the test. Antigens may also be titrated against sera.

Two important parameters of serological tests are sensitivity and specificity. Sensitivity refers to the ability of the test to detect even very minute quantities of antigen or antibody. When a test is highly sensitive, false negative results will be absent or minimal. Specificity refers to the ability of the test to detect reactions between homologous antigens and antibodies only, and with no other. In a highly specific test, false positive reactions will be absent or minimal. In general, sensitivity and specificity of a test will be in inverse proportion.

PRECIPITATION REACTION

When a soluble antigen combines with its antibody in the presence of electrolytes (NaCl) at a suitable temperature and pH, the antigen-antibody complex forms an insoluble precipitate. When instead of sedimenting, the precipitate

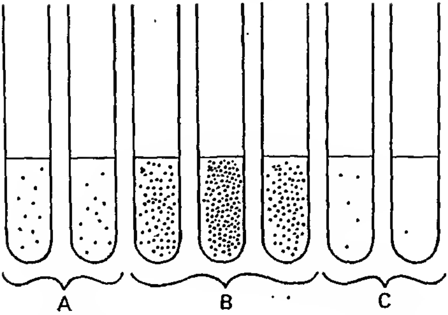

Fig. 13.1 A quantitative precipitation test showing A prozone (zone of antibody excess), B zone of equivalence, C zone of antigen excess

remains suspended as floccules, the reaction is known as flocculation. Precipitation can take place in liquid media or in gels such as agar, agarose or polyacrylamide.

The amount of precipitate formed is greatly influenced by the relative proportions of antigens and antibodies. If to the same amount of antiserum in different tubes, increasing quantities of antigens are added, precipitation will be found to occur most rapidly and abundantly in one of the middle tubes, in which the antigen and the antibody are present in optimal or equivalent proportion. In the preceding tubes, in which the antibody is in excess, and in the later tubes, in which the antigen is in excess, the precipitation will be weak or even absent. For a given antigen-antibody system, the optimal or equivalent ratio will be constant, irrespective of the quantity of the reactants. If the amounts of precipitate in the different tubes are plotted on a graph, the resulting curve will have three phases: an ascending part (prozone or zone of antibody excess), a peak (zone of equivalence) and a descending part (postzone or zone of antigen excess) (Fig. 13.1). This is called the zone phenomenon. Zoning occurs in agglutination and some other serological reactions also. The prozone is of importance in clinical serology, as sometimes sera rich in antibody may give a false negative precipitation or agglutination result, unless several dilutions are tested.

Mechanism of precipitation

Marrack (1934) proposed the lattice hypothesis to explain the mechanism of precipitation. According to this concept, which is supported by considerable experimental evidence and is now widely accepted, multivalent antigens combine with bivalent antibodies in varying proportions, depending on the antigen-antibody ratio in the reacting mixure. Precipitation results when a large lattice is formed consisting of alternating antigen and antibody molecules. This is possible only in the zone of equivalence. In the zones of antigen or antibody excess, the lattice does not

Tube Test: Kahn Test

enlarge, as the valencies of the antibody and the antigen, respectively, are fully satisfied (Fig. 13.2). The lattice hypothesis holds good for agglutination also.

Applications of precipitation reaction

The precipitation test may be carried out either as a qualitative or as a quantitative test. It is very sensitive for detecting antigens and as little as 1μg of protein can be detected by precipitation tests. It, therefore, finds forensic application in the identification of blood and seminal stains, and in testing for food adulterants. Precipitation is relatively less sensitive for the detection of antibodies.

The following types of precipitation and flocculation tests are in common use:

Ring test: This, the simplest type of precipitation test, consists of layering the antigen solution over a column of antiserum in a narrow tube. A precipitate forms at the junction of the two liquids. Examples of ring precipitation tests are the C-reactive protein test, Ascoli's thermoprecipitin test and the grouping of streptococci by the Lancefield technique.

Slide test: When a drop each of the antigen and antiserum are placed on a slide and mixed by shaking, floccules appear. The VDRL test for syphilis is an example of slide flocculation.

Tube test: The Kahn test for syphilis is an example of a tube flocculation test. A quantitative tube flocculation test is employed for the standardisation of toxins and toxoids. Serial dilutions of the toxin/toxoid are added to the tubes containing a fixed quantity of the antitoxin. The amount of toxin or toxoid that flocculates optimally with one unit of the antitoxin is defined as an Lf dose.

Immunodiffusion (Precipitation in gel): There are several advantages in allowing precipitation to occur in a gel rather than in a liquid medium. The reaction is visible as a distinct band of precipitation, which is stable and can be stained for preservation, if necessary. As each antigen-anti-

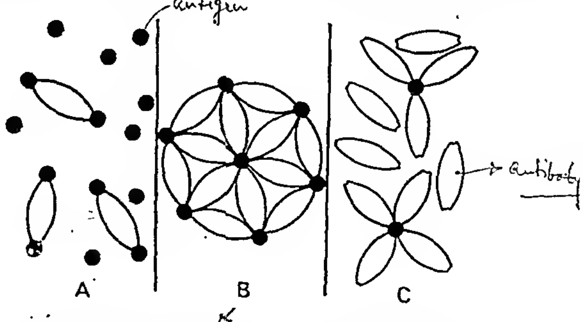

Fig. 13.2 Mechanism of precipitation by lattice formation. In A (antigen excess) and C (antibody excess), lattice formation does not occur. In B (zone of equivalence), lattice formation (and precipitation occurs optimally. The dark spheres indicate antigen and the spindles bivalent antibody molecules.

body reaction gives rise to a line of precipitation, the number of different antigens in the reacting mixture can be readily observed. Immunodiffusion also indicates identity, cross reaction and nonidentity between different antigens

Immunodiffusion is usually performed in a soft (1 %) agar gel. Different modifications of the test are available:

1. *Single diffusion in one dimension (Oudin procedure)* The antibody is incorporated in agar gel in a test tube and the antigen solution is layered over it. The antigen diffuses downward through the agar gel, forming a line of precipitation that appears to move downwards. This is due to the precipitation formed at the advancing front of the antigen, being dissolved as the concentration of antigen at the site increases due to diffusion. The number of bands indicates the number of different antigens present.

2. *Double diffusion in one dimension (Oakley-Fulthorpe procedure):* Here, the antibody is incorporated in gel, above which is placed a column of plain agar. The antigen is layered on top of this. The antigen and antibody move towards each other through the intervening column of plain agar and form a band of precipitate where they meet at optimum proportion.

3. *Single diffusion in two dimensions (Radial immunodiffusion)*: Here the antiserum is incorporated in agar gel poured on a flat surface (slide or Petri dish). The antigen is added to the wells cut on the surface of the gel. The antigen diffuses radially from the well and forms ring-shaped bands of precipitation (halos) concentrically around the well. The diameter of the halo gives an estimate of the concentration of the antigen. This method has been employed for the estimation of the immunoglobulin classes in sera. It has

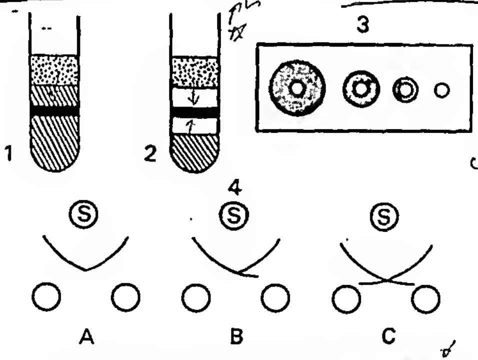

Fig 13 3 Different types of immunodiffusion: 1 Single diffusion in one dimension (Oudin) 2 Double diffusion in one dimension (Oakley-Fulthorpe) 3. Radial immunodiffusion 4 Double diffusion in two dimensions (Ouchterlony), showing reaction of identity (A), partial identity or relatedness (B), and lack of relatedness (C). The wells marked S contain antiserum and unmarked wells contain antigens

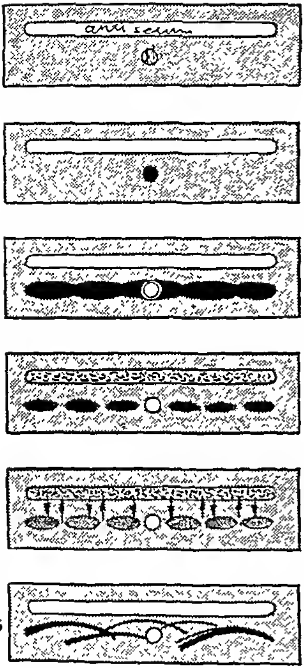

Fig. 13.4 Immunoelectrophoresis.
1. Semisolid agar layered on the glass slide. A well for antigen and a trough for antiserum cut out of agar. 2. Antigen well filled with human serum. 3. Serum separated by electrophoresis. 4. Antiserum trough filled with antiserum to whole human serum. 5. Serum and antiserum allowed to diffuse into agar. 6. Precipitin lines form for individual serum proteins

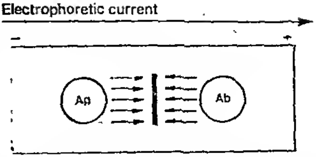

Fig. 13.5 Counterimmunoelectrophoresis (CIE). Antigen and antibody are driven together by an electric current and a precipitin line forms

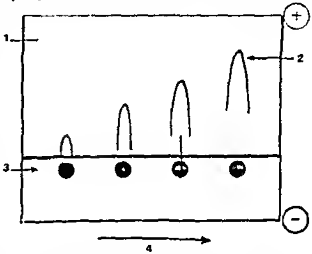

Fig. 13.6 Rocket electrophoresis. Antigen is driven into gel containing antibody. 1. Antibody in agarose gel. 2. Precipitin arcs. 3. Antigen wells. 4. Increasing antigen concentration

also been used for screening sera for antibodies to influenza viruses.

4. Double diffusion in two dimensions (Ouchterlony procedure): This is the immunodiffusion method most widely employed and helps to compare different antigens and antisera directly. Agar gel is poured on a slide and wells are cut using a template. The antiserum is placed in the central well and different antigens in the surrounding wells. If two adjacent antigens are identical, the lines of precipitate formed by them will fuse. If they are unrelated, the lines will cross each other. Cross reaction or partial identity is indicated by spur formation (Fig. 13.3).

Double in Two dimension – small pox & Elek Test for dyptheria

This method was a routine technique for the diagnosis of smallpox. When extracts of smallpox lesions are tested against the antiserum, precipitation lines can be seen within 2-6 hours. The reaction may be speeded up by electrophoresis (counterimmunoelectrophoresis) and a positive result obtained within about 60 minutes.

A special variety of double diffusion in two dimensions is the Elek test for toxigenicity in diphtheria bacilli. When diphtheria bacilli are streaked at right angles to a filter paper strip carrying the antitoxin implanted on a plate of suitable medium, arrowhead shaped lines of precipitation appear on incubation, if the bacillus is toxigenic.

5. *Immunoelectrophoresis:* The resolving power of immunodiffusion was greatly enhanced when immunoelectrophoresis was devised by Grabar and Williams (1953). This consists of two steps. The first step is agar electrophoresis of the antigen. Rectangular troughs are then cut in the agar, on either side, parallel to the direction of electrophoretic migration, and are filled with the antiserum. By diffusion, lines of precipitation develop with each of the separated components of the antigen. By immunoelectrophoresis, over 30 different antigens can be identified in human serum. The technique is particularly useful in testing for normal and abnormal serum proteins (Fig. 13.4).

Electroimmunodiffusion

The development of precipitin lines can be speeded up by electrically driving the antigen and antibody. Various methods have been described combining electrophoresis with diffusion. Of these, one dimensional double electroimmunodiffusion (counterimmunoelectrophoresis) and one dimensional single electroimmunodiffusion (rocket electrophoresis) are used frequently in the clinical laboratory.

1. *Counterimmunoelectrophoresis* (CIE, counter current immunoelectrophoresis): This involves simultaneous electrophoresis of the antigen and antibody in gel in opposite directions resulting in precipitation at a point between them (Fig. 13.5). This method produces visible precipitation lines within thirty minutes and is ten times more sensitive than the standard double diffusion techniques. The clinical applications are for detecting various antigens such as HBs Ag, Alpha fetoprotein in serum and specific antigens of cryptococcus in the cerebrospinal fluid.

2. *One dimensional single electroimmunodiffusion* (Rocket electrophoresis): The main application of this technique is for quantitative estimation of antigens. The antiserum to the antigen to be quantitated is incorporated in agarose and gelled on the glass slide. The antigen, in increasing concentrations, is placed in wells punched in the set gel. The antigen is then electrophoresed into the antibody-containing agarose (Fig. 13.6). The pattern of immuno-precipitation resembles a rocket and hence the name.

A variant of this is Laurell's two dimensional electrophoresis. In this technique, the antigen mixture is first electrophoretically separated in a direction perpendicular to that of the final rocket stage. By this method one can quantitate each of several antigens in a mixture (Fig. 13.7).

AGGLUTINATION REACTION

When a particulate antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated. Agglutination is more sensitive than precipitation for the detection of antibodies. The same principles govern agglutination and precipitation. Agglutination occurs optimally when antigens and antibodies react in equivalent proportions. The zone phenomenon may be seen when either an antibody or an antigen is in excess. 'Incomplete' or 'monovalent' antibodies do not cause agglutination, though they combine with the antigen. They may act as 'blocking' antibodies, inhibiting agglutination by the complete antibody added subsequently.

Applications of agglutination reaction

Slide agglutination: When a drop of the appropriate antiserum is added to a smooth, uniform suspension of a particulate antigen in a drop of saline on a slide or a tile, agglutination takes place. A positive result is indicated by the clumping together of the particles and the clearing of the drop. Mixing the antigen and the antiserum with a loop or by gently rocking the slide facilitates the reaction. Depending on the titre of the serum, agglutination may occur instantly or within seconds. Clumping occurring after a minute may be due to drying of the fluid and should be disregarded. It is essential to have on the same slide a control consisting of the antigen suspension in saline, without the antiserum, to ensure that the antigen is not autoagglutinable. Agglutination is usually visible to the naked eye, but may sometimes require confirmation under the microscope. Slide agglutination is a routine procedure for the identication of many bacterial isolates from clinical specimens. It is also the method used for blood grouping and cross matching.

Tube agglutination: This is a standard quantitative method for measurement of antibodies. When a fixed volume of a particulate antigen suspension is added to an equal volume of serial dilutions of an antiserum in test tubes, the agglutination titre of the serum can be estimated. Tube agglutination is routinely employed for the serological diagnosis of typhoid, brucellosis and typhus fevers.

In the Widal test used in typhoid, two types of antigens are used, the 'H' or the flagellar antigen and the 'O' or the somatic antigen of the typhoid bacillus. The H antigen is a formolised suspension of the bacillus and on combination with the antibody, forms large, loose, fluffy clumps resembling wisps of cottonwool. Conical Dreyer's tubes are used for H agglutination. The O antigen is prepared by treating the bacterial suspension with alcohol. It forms tight, compact deposits resembling chalk powder. Round-bottomed Felix tubes are used for O agglutination. Agglutinated bacilli spread out in a disc-like pattern at the bottom of the tubes.

The tube agglutination test for brucellosis may

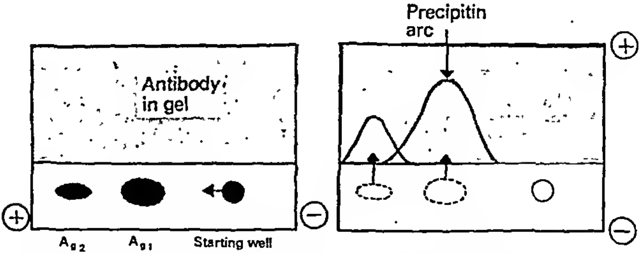

Fig. 13 7 Laurell's variant of rocket electrophoresis (Two dimensional immunoelectrophoresis). First run, antigens are separated on the basis of electrophoretic mobility. The second run at right angles to the first drives the antigens into the antiserum containing gel to form precipitation peaks. The area of the peak is proportional to the concentration of the antigen

be complicated by the prozone phenomenon and the presence of 'blocking' antibodies. Several dilutions of the serum should be tested to prevent false negative results due to prozone. Incomplete or blocking antibodies may be detected by doing the test in hypertonic (5 %) saline or albumin saline, or more reliably by the antiglobulin (Coombs) test. Incomplete antibodies are also met with in sera containing Rh antibodies. Agglutination can often be obtained if Rh-positive erythrocytes, treated with proteolytic enzymes (papain, bromelain), are used as the antigen.

The Weil-Felix reaction for serodiagnosis of typhus fevers is a heterophile agglutination test and is based on the sharing of a common antigen between typhus rickettsiae and some strains of proteus bacilli. Another example of the heterophile agglutination test is the *Streptococcus MG* agglutination test for the diagnosis of primary atypical pneumonia.

Examples of the agglutination tests using red cells as antigens are the Paul-Bunnell test and the cold agglutination test. The former is based on the appearance in the sera of infectious mononucleosis patients, of agglutinins to sheep erythrocytes, which are adsorbed by ox red cells but not by guinea pig kidney extract. The cold agglutination test is positive in primary atypical pneumonia. The patients' sera agglutinate human O group erythrocytes at 4°C, the agglutination being reversible at 37°C.

The antiglobulin (Coombs) test: The antiglobulin test was devised by Coombs, Mourant and Race (1945) for the detection of anti-Rh antibodies that do not agglutinate Rh-positive erythrocytes in saline. When sera containing incomplete anti-Rh antibodies are mixed with Rh-positive red cells, the antibody globulin coats the surface of the erythrocytes, though they are not agglutinated. When such erythrocytes coated with the antibody globulin are washed free of all unattached protein and treated with a rabbit antiserum against human gamma globulin (antiglobulin or Coombs serum), the cells are agglutinated. This is the principle of the antiglobulin test (Fig. 13.8).

The Coombs test may be of the direct or the indirect type. In the direct Coombs test, the sensitisation of the erythrocytes with incomplete antibodies takes place *in vivo*, as in the haemolytic disease of the newborn due to Rh incompatibility. When the red cells of erythroblastotic infants are washed free of unattached protein and then mixed with a drop of Coombs serum, agglutination results. For unknown reasons, the direct Coombs test is often negative in haemolytic disease due to ABO incompatibility.

In the indirect Coombs test, sensitisation of red cells with the anitbody globulin is performed *in vitro*. Originally employed for detection of anti-Rh antibodies, the Coombs test is useful for demonstrating any type of incomplete or nonagglutinating antibody, as, for example, in brucellosis.

Passive agglutination test: The only difference between the requirements for the precipitation and agglutination tests is the physical nature of the antigen. By attaching soluble antigens to the surface of carrier particles, it is possible to convert precipitation into agglutination tests, which are more convenient and more sensitive for detection of antibodies. Such tests are known as passive agglutination tests.

The carrier particles commonly used are red cells, latex particles or bentonite. Human or sheep erythrocytes adsorb a variety of antigens. Polysaccharide antigens may be adsorbed by simple mixing with the cells. For adsorption of protein antigens, tanned red cells are used.

A special type of passive haemagglutination test is the Rose-Waaler test. In rheumatoid arthritis, an autoantibody (RA factor) appears in the serum, which acts as an antibody to gamma globulin. The RA factor is able to agglutinate red cells coated with globulins. The antigen used for the test is a suspension of sheep erythrocytes sensitised with a subagglutinating dose of rabbit antisheep erythrocyte antibody (amboceptor).

Polystyrene latex, which can be manufactured as uniform spherical particles, 0.8 – 1μ in diameter, can adsorb several types of antigens

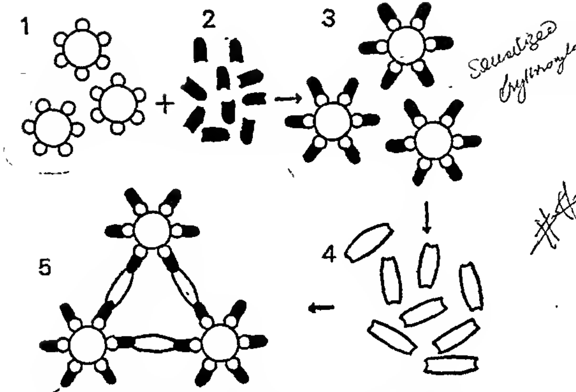

Fig 13.8 Antiglobulin (Coombs) test Rh positive erythrocytes (1) are mixed with incomplete antibody (2) The antibody coats the cells (3) but being incomplete, cannot produce agglutination On addition of antiglobulin serum (4) which is complete antibody to immunoglobulin agglutination takes place

Latex agglutination tests (latex fixation tests) are widely employed in the clinical laboratory for detection of hepatitis B, ASO, CRP, RA factor, HCG and many other antigens.

Passive agglutination tests are very sensitive and yield high titres. When instead of the antigen, the antibody is adsorbed to carrier particles in tests for estimation of antigens, the technique is known as reversed passive agglutination.

COMPLEMENT FIXATION TESTS (CFT)

Complement takes part in many immunological reactions and is absorbed during the combination of antigens with their antibodies. In the presence of the appropriate antibodies, complement lyses erythrocytes, kills and, in some cases, lyses bacteria, immobilises motile organisms, promotes phagocytosis and immune adherence and contributes to tissue damage in certain types of hypersensitivity.

The ability of antigen-antibody complexes to 'fix' complement is made use of in the complement fixation test (CFT). This is a very versatile and sensitive test, applicable with various types of antigens and antibodies and capable of detecting as little as 0.04 μg of antibody nitrogen and 0.1 μg of antigen. CFT is a complex procedure consisting of two steps and five reagents — antigen, antibody, complement, sheep erythrocytes and amboceptor (rabbit antibody to sheep red cells). Each of these reagents has to be separately standardised.

The antigen may be soluble or particulate. The antiserum should be heated at 56°C (inactivated) for half an hour before the test to destroy any complement activity the serum may have and also to remove some nonspecific inhibitors of comple-

ment present in some sera (anticomplementary activity). The source of complement is guinea pig serum. As complement activity is heat labile, the serum should be freshly drawn, or preserved either in the lyophilised or frozen state or with special preservatives, as in Richardson's method. The guinea pig serum should be titrated for complement activity. One unit or minimum haemolytic dose (MHD) of complement is defined as the highest dilution of the guinea pig serum that lyses one unit volume of washed sheep erythrocytes in the presence of excess of haemolysin (amboceptor) within a fixed time (usually 30 or 60 minutes) at a fixed temperature (37°C). The amboceptor should be titrated for haemolysin activity. One MHD of haemolysin is defined as the least amount (or highest dilution) of the inactivated amboceptor that lyses one unit volume of washed sheep erythrocytes in the presence of excess complement within a fixed time (usually 30 or 60 minutes) at a fixed temperature (37°C). The diluent used for the titrations and for CFT is physiological saline with added calcium and magnesium ions.

The classical example of CFT is the Wassermann reaction, formerly the routine method for the serodiagnosis of syphilis. The test consists of two steps. In the first, the inactivated serum of the patient is incubated at 37°C for one hour with the Wassermann antigen and a fixed amount (two units) of guinea pig complement. If the serum contains syphilitic antibody, the complement will be utilised during the antigen-antibody interaction. If the serum does not contain the antibody, no antigen-antibody reaction occurs and, therefore, the complement will be left intact. Testing for complement in the post-incubation mixture will, therefore, indicate whether the serum had antibodies or not. This constitutes the second step in the test and consists of adding sensitised cells (sheep erythrocytes coated with 4 MHD haemolysin), and incubating at 37°C for 30 minutes. Lysis of the erythrocytes indicates that complement was not fixed in the first step and, therefore, the serum did not have the antibody (negative CFT). Absence of erythrocyte lysis indicates that the complement was used up in the first step, and, therefore, the serum contained the antibody (positive CFT) (Fig. 13.9).

Appropriate controls should be put up, including the following: antigen and serum controls to ensure that they are not anticomplementary, complement control to ensure that the desired amount of complement had been added, and cell

I ANTIGEN + TEST SERUM
(CONTAINS ANTIBODY)
+ COMPLEMENT
— COMPLEMENT FIXED

+ HAEMOLYTIC SYSTEM
RESULT – NO HAEMOLYSIS
POSITIVE CF TEST

II ANTIGEN + TEST SERUM
(CONTAINS NO ANTIBODY)
+ COMPLEMENT
COMPLEMENT NOT FIXED

+ HAEMOLYTIC SYSTEM
RESULT – HAEMOLYSIS
NEGATIVE CF TEST.

Fig. 13.9 Complement fixation test.

control to see that sensitised erythrocytes do not undergo lysis in the absence of complement.

Indirect complement fixation test: Certain avian (e.g., duck, turkey, parrot) and mammalian (e.g., horse, cat) sera do not fix guinea pig complement. When such sera are to be tested, the indirect complement fixation test may be employed. Here the test is set in duplicate and after the first step, the standard antiserum known to fix complement is added to one set. If the test serum contained antibody, the antigen would have been used up in the first step and therefore the standard antiserum added subsequently would not be able to fix complement. Therefore, in the indirect test, haemolysis indicates a positive result.

Conglutinating complement absorption test: For systems which do not fix guinea pig complement, an alternative method is the conglutinating complement absorptinn test. This uses horse complement which is nonhaemolytic. The indicator system is sensitised sheep erythrocytes mixed with bovine serum. Bovine serum contains a beta globulin component called conglutinin, which acts as antibody to complement. Therefore, conglutinin causes agglutinatinn of sensitised sheep erythrocytes (conglutination) if they have combined with complement. If the horse complement had been used by the antigen-antibody interaction in the first step, agglutination nf sensitised cells will not occur.

Other complement dependent serological tests: When some bacteria (e.g., *Vibrio cholerae*, *Treponema pallidum*) react with the specific antibody in the presence of complement and particulate materials such as erythrocytes or platelets, the bacteria are aggregated and adhere to the cells. This is known as *immune adherence*. The *immobilisation test* is another complement dependent reaction. In the '*Treponema pallidum* immobilisation test', which is a highly specific test for the serodiagnosis of syphilis, the test serum is mixed with a live motile suspension of *T. pallidum* in the presence of complement. On incubation, the specific antibody inhibits the motility of the treponemes. *Cytolytic* or *cytocidal* tests also are complement dependent. When a suitable live bacterium, such as the cholera vibrio, is mixed with its antibody in the presence of complement, the bacterium is killed and lysed. This forms the basis of the vibriocidal antibody test for the measurement of anticholera antibodies.

NEUTRALISATION TESTS

Virus neutralisation tests: Neutralisation of viruses by their nntibodies can be demonstrated in various systems. Neutralisation of bacteriophages can be demonstrated by the plaque inhibition test. When bacteriophages are seeded in appropriate dilution on lawn cultures of susceptible bacteria, plaques of lysis are produced. Specific antiphage serum inhibits plaque formation. Neutralisation of animal viruses can be demonstrated in three systems — animals, eggs and tissue culture.

Toxin neutralisation: Bacterial exotoxins are good antigens and induce the formation of neutralising antibodies (antitoxins) which are important clinically, in protection against and recovery from diseases such as diphtheria and tetanus. The toxicity of endotoxins is not neutralised by antisera.

Toxin neutralisation can be tested *in vivo* or *in vitro*. Neutralisation tests in animals consist of injecting into them toxin-antitoxin mixtures and estimating the least amount of antitoxin that prevents death or disease in the animal. With the diphtheria toxin, which in small doses causes a cutaneous reaction, neutralisation tests can be done on the human skin. The Schick test is based on the ability of circulating antitoxin to neutralise the diphtheria toxin given intradermally, and indicates the immunity or susceptibility to the disease. Toxin neutralisation *in vitro* depends on the inhibition of some demonstrable toxic effect. An example is the antistreptolysin O test, in which antitoxin present in patients' sera neutralises the haemolytic activity of the streptococcal O haemolysin.

OPSONISATION

The name 'opsonin' was originally given by Wright (1903) to a heat labile substance present in fresh normal sera, which facilitated phagocytosis. This factor was subsequently identified as complement. A heat stable serum factor with similar activity was called 'bacteriotropin'. This appears to be specific antibody. The term opsonin is now generally used to refer to both these factors. Wright used the 'opsonic index' to study the progress of resistance during the course of diseases. The opsonic index was defined as the ratio of the phagocytic activity of the patient's blood for a given bacterium, to the phagocytic activity of blood from a normal individual. It was measured by incubating fresh citrated blood with the bacterial suspension at 37°C for 15 minutes and estimating the average number of phagocytosed bacteria per polymorphonuclear leucocyte (phagocytic index) from stained blood films.

IMMUNOFLUORESCENCE

Fluorescence is the property of absorbing light rays of one particular wavelength and emitting rays with a different wavelength. Fluorescent dyes show up brightly under ultraviolet light as they convert ultraviolet into visible light. Coons and his colleagues (1942) showed that fluorescent dyes can be conjugated to antibodies and that such 'labelled' antibodies can be used to locate

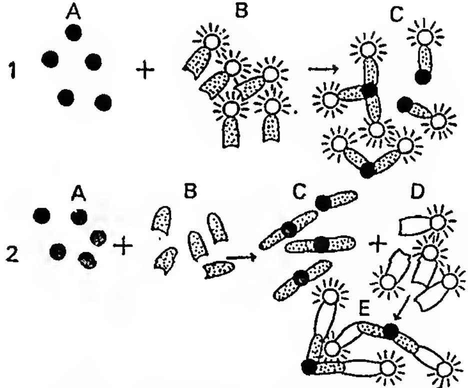

Fig. 13.10 Immunofluorescence. 1. Direct Test. Antigen (A) is mixed with fluorescent-conjugated antibody (B). The antigen antibody complex is fluorescent (C). 2. Indirect test. Antigen (A) is mixed with antibody (B). The antigen antibody complex (C) is treated with fluorescent conjugated antiglobulin serum (D). The final product is fluorescent (E).

and identify antigens in tissues. This 'fluorescent antibody' or immunofluorescence technique has several diagnostic and research applications.

In its simplest forms (direct immunofluorescence test), it can be used for the identification of bacteria, viruses or other antigens, using the specific antiserum labelled with a fluorescent dye. For example, direct immunofluorescence is now routinely used as a sensitive method of diagnosing rabies, by detection of the rabies virus antigens in brain smears. A disadvantage of this method is that separate fluorescent conjugates have to be prepared against each antigen to be tested. The 'indirect immunofluorescence test' overcomes this difficulty by using an antiglobulin fluorescent conjugate. An example is the fluorescent treponemal antibody test for the diagnosis of syphilis. Here a drop of the test serum is placed on a smear of *T. pallidum* on a slide and after incubation, the slide is washed well to remove all free serum, leaving behind only antibody globulin, if present, coated on the surface of the treponemes. The smear is then treated with a fluorescent labelled antiserum to human gamma globulin. The fluorescent conjugate reacts with antibody globulin bound to the treponemes. After washing away all the unbound fluorescent conjugate, when the slide is examined under ultraviolet illumination, the treponemes will be seen as bright objects against a dark background, if the test is positive. If the serum does not have antitreponemal antibody, there will be no globulin coating on the treponemes and, therefore, they will not take on the fluorescent conjugate. A single antihuman globulin fluorescent conjugate can be employed for detecting human antibody to any antigen (Fig. 13.10).

Fluorescent dyes may also be conjugated with complement. Labelled complement is a versatile tool and can be employed for the detection of antigen or antibody. Antigens also take fluorescent labelling, but not as well as antibodies do. For detection of antibodies by immunofluorescence, the 'sandwich' technique can be employed. The antibody is first allowed to react with unlabelled antigen, which is then treated with fluorescent labelled antibody, thus forming a sandwich, the antigen being in the middle, with labelled and unlabelled antibody on either side.

The fluorescent dyes commonly used are fluorescein isothiocyanate and lissamine rhodamine, exhibiting blue-green and orange-red fluorescence, respectively. By combining the specificity of serology with the localising capacity of histology, immunofluorescence helps in the visualisation of antigen-antibody reactions *in situ*. It is thus an immuno-histochemical technique. The major disadvantage of the technique is the frequent occurrence of nonspecific fluorescence in tissues and other materials.

RADIOIMMUNOASSAY (RIA)

Besides fluorescent dyes, many other distinctive 'labels' also can be conjugated to antigens and antibodies. The most commonly used labels are radioisotopes and enzymes. A variety of tests have been devised for measurement of antigens and antibodies using such labelled reactants. The term *binder-ligand assay* has been used for these reactions. The substance (antigen) whose concentration is to be determined is termed the *analyte* or *ligand*. The binding protein (ordinarily the antibody) which binds to the ligand is called the *binder*. The first reaction of this type was the *radioimmunoassay* (RIA) described by Berson and Yallow in 1959. RIA permits the measurement of analytes upto picogram (10^{-12}g) quantities. RIA and its modifications have versatile applications in various areas of biology, and medicine, including the quantitation of hormones, drugs, tumour markers, IgE and viral antigens. The importance of RIA was acknowledged by awarding the Nobel Prize for its discovery to Yallow in 1977.

RIA is a competitive binding assay in which fixed amounts of antibody and radiolabelled antigen react in the presence of unlabelled antigen. The labelled and unlabelled antigens compete for the limited binding sites on the antibody. This competition is determined by the level of the

unlabelled (test) antigen present in the reacting system. After the reaction, the antigen is separated into the 'free' and 'bound' fractions and their radioactive counts measured. The concentration of the test antigen can be calculated from the ratio of the bound and total antigen labels, using a standard dose response curve.

For any reacting system, the *standard dose response or calibrating curve* has to be prepared first. This is done by running the reaction with fixed amounts of antibody and labelled antigen, and varying known amounts of unlabelled antigen. The ratios of bound: total labels (B:T ratio) plotted against the analyte concentrations give the standard calibration curve. The concentration of antigen in the test sample is computed from the B:T ratio of the test by interpolation from the calibration curve.

Enzyme-linked Immunosorbent assay (ELISA)

This is a simple and versatile technique, which is nearly as sensitive as radioimmunoassay and needs only microlitre quantities of test reagents. ELISA has found application for the detection of a variety of antibodies and antigens, such as hormones, toxins and viruses. The test may be done in polystyrene tubes (macro-ELISA) or polyvinyl microtitre plates (micro-ELISA). The principle of the test for the detection of antigens can be illustrated by outlining its application for the detection of the rotavirus antigen in faeces.

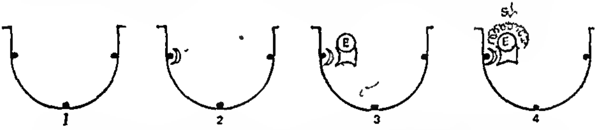

Fig 13.11 ELISA for detection of rotavirus in faeces. 1 Microassay plate coated with goat antibody to rotavirus (•) When faecal suspension is added and incubated rotavirus if present, will adsorb to the coated antibody. 3. After thorough washings add guinea pig antirotavirus antibody conjugated with alkaline phosphatase enzyme ( ) If rotavirus is present the conjugate will complex with it 4 After washing add the substrate, paranitrophenyl phosphate 5 If the enzyme conjugate is present, the substrate will be split to form yellow coloured product This indicates a positive test In a negative test there will be no colour formation

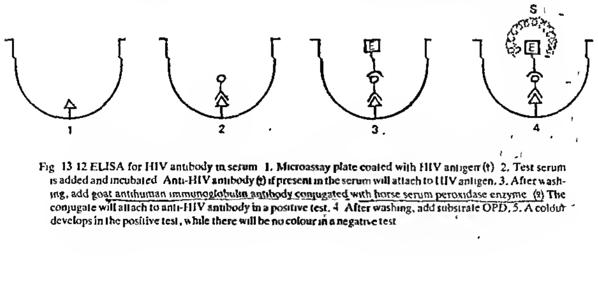

Fig 13.12 ELISA for HIV antibody in serum 1. Microassay plate coated with HIV antigen 2. Test serum is added and incubated Anti-HIV antibody if present in the serum will attach to HIV antigen. 3. After washing, add goat antihuman immunoglobulin antibody conjugated with horse serum peroxidase enzyme The conjugate will attach to anti-HIV antibody in a positive test. 4 After washing, add substrate OPD. 5. A colour develops in the positive test, while there will be no colour in a negative test

The wells of a microtitre plate are coated with goat antirotavirus antibody. After thorough washing, the faecal samples to be tested are added and incubated overnight at 4°C or for two hours at 37°C. Suitable positive and negative controls are also put up. The wells are washed and guinea pig anttirotavirus antiserum, labelled with alkaline phosphatase, added and incubated at 37°C for one hour. After washing, a suitable substrate (paranitrophenyl phosphate) is added and held at room temperature till the positive controls show the development of a yellow colour. The phosphatase enzyme splits the substrate to yield a yellow compound.

If the test sample contains rotavirus, it is absorbed to the antibody coating the wells. When the enzyme labelled antibody is added subsequently, it is in turn absorbed. The presence of residual enzyme activity, indicated by the development of yellow colour, therefore, denotes a positive test (Fig. 13.11). If the sample is negative, there is no colour change.

The detection of antibody by ELISA can be illustrated by the anti-HIV antibody test. Purified Inactivated HIV antigen is absorbed onto microassay plate wells. Test serum diluted in buffer is added to the well and incubated at 37°C for 30 minutes. The well is then thoroughly washed. If the serum contains anti-HIV antibody, it will form a stable complex with the HIV antigen on the plate. A goat antihuman immunoglobulin antibody conjugated with horse radish peroxidase enzyme is added and incubated for 30 minutes. After thorough washing, the substrate O-phenylene diamine dihydrochloride is added and after 30 minutes, the colour that develops is read using a microassay plate reader at 492 n.m Positive and negative controls should invariably be used with test sera (Fig. 13.12).

Several variations of the ELISA technique have been developed to provide simple diagnostic tests, including card and dipstick methods suitable for clinical laboratory and bedside applications.

IMMUNOELECTRONMICROSCOPIC TESTS

Immunoelectronmicroscopy: When viral particles mixed with the specific antisera are observed under the electron microscope, they are seen to be clumped. This finds application in the study of some viruses such as hepatitis A virus and the viruses causing diarrhoea.

Immunoferritin test: Ferritin (an electrondense substance from horse spleen) can be conjugated with antibody, and such labelled antibody reacting with an antigen can be visualised under the electron microscope.

Immunoenzyme test: Some stable enzymes, such as peroxidase, can be conjugated with antibodies. Tissue sections carrying the corresponding antigens are treated with peroxidase labelled antisera. The peroxidase bound to the antigen can be visualised under the electron microscope, by micro-histochemical methods. Some other enzymes, such as glucose oxidase, phosphatases and tyrosinase, may also be used in immunoenzyme tests.

Further Reading

Of 8

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C complement ... c/g act of biological activity induced by both ... all the complement ...

14 The Complement System

The term 'complement' (C) refers to a system of factors occurring in normal serum, that are activated characteristically by antigen-antibody interaction, and subsequently mediate a number of biologically significant consequences.

Towards the end of the 19th century, it was noticed that bactericidal, bacteriolytic and haemolytic action of the appropriate antibodies required the participation of a heat labile component present in the normal sera of man and animals. Buchner (1889) was the first to observe that the bactericidal effect of serum was destroyed by heating at 55° C for one hour. Pfieffer (1894) discovered that cholera vibrios were lysed when injected intraperitoneally into specifically immunised guinea pigs (bacteriolysis *in vivo* or Pfieffer's phenomenon). Bordet (1895) extended these observations and established that immune bacteriolysis and haemolysis required two factors — the heat stable antibody and a heat labile factor, which was called *alexine*. This term has been replaced by the present name *complement* which was coined by Ehrlich, because it complemented the action of antibody.

Bordet and Gengou (1901) described the complement fixation test, using the haemolytic indicator system, as a sensitive serological reaction. This found wide application, and the Wassermann complement fixation test for syphilis became one of the most popular serological tests. For the next half century, interest in complement remained confined to its use as a tool in serological reactions. Since then the structural and functional complexities of the complement system have been defined and its role as a mediator and amplifier of many immune and inflammatory reactions recognised. The complement system belongs to the group of biological effector mechanisms (called *triggered enzyme cascades*) which also includes the coagulation, fibrinolytic and kinin systems.

The complement system consists of at least 20 chemically and immunologically distinct serum proteins comprising the complement components, the properdin system and the control proteins.

Components of complement

Complement is not a single substance, but a complex of nine different fractions called C1 to C9. The fraction C1 occurs in serum as a calcium ion dependent complex, which on chelation with EDTA yields three protein subunits called C1q, r and s. Thus C is made up of a total of 11 different proteins. Complement fractions are named C1 to C9 in the sequence of the cascading reaction, except that C4 comes after C1, before C2.

Complement constitutes 10–15 per cent of human serum globulins. Though some of its components are heat stable, C as a whole is heat labile, undergoing spontaneous denaturation slowly at room temperature and in 30 minutes at 56°C. Serum deprived of its C activity by heating at 56°C for 30 minutes is said to be 'inactivated'. C ordinarily does not combine with either free antigen or antibody, but only with antibody that has combined with its antigen. Not all classes of antibodies can fix complement. The C binding site is located on the CH2 homology of the *Fc* portion of IgM and IgG (1,2 and 3) molecules

only, and is expressed only when the antibody has combined with its antigen.

Complement is normally present in the body in an inactive form, but when its activity is induced by antigen-antibody combination or other stimuli, C components react in a specific sequence as a cascade. Basically, the C cascade is a series of reactions in which the preceding components act as enzymes on the succeeding components, cleaving them into dissimilar fragments. The larger fragments usually join the cascade. The smaller fragments which are released often possess biological effects which contribute to defence mechanisms by amplifying the inflammatory process, increasing vascular permeability, inducing smooth muscle contraction, causing chemotaxis of leucocytes; promoting virus neutralisation, detoxyfying endotoxins and effecting the release of histamine from mast cells.

The C cascade can be triggered off by two parallel but independent mechanisms or pathways which differ only in the initial steps. Once C3 activation occurs, the subsequent steps are common in both pathways, which have been called the *classical C pathway* and the *alternative* or *properdin* pathway.

Classical C pathway

The chain of events in which C components react in a specific sequence, following activation by antigen-antibody combination, and typically culminating in immune cytolysis, is known as the classical C pathway.

The model traditionally used to explain C activity in immune cytolysis is the lysis of erythrocyte sensitised by its antibody. The erythrocyte (E)-antibody (A) complex is called EA and when C components are attached to EA, the product is called EAC, followed by the components that have reacted (e.g., EAC 14235 or EAC1-5). When a C component acquires enzymatic or other demonstrable biological activity, it is indicated by a bar over the component number, e.g., the enzymatically activated C1 is shown as $\overline{C1}$. Fragments cleaved from C components during the cascade are indicated by small letters (e.g., C3a, C3b). Inactivate forms of C components are indicated by the suffix 'i' (e.g., C4i).

Immune cytolysis is initiated by the binding of C1 to EA. The recognition unit of C1 is C1q, which reacts with the *Fc* piece of bound IgM or IgG molecules. This, in turn sequentially activates C1 r and s. The activated C1s ($\overline{C1}$) is an esterase which acts on C4, splitting it into C4a and b, of which C4b joins the cascade. $\overline{C14b}$ in presence of magnesium ions cleaves C2. C2a, the larger fragment, remains attached to $\overline{C4}$ to form $\overline{C42}$ which is an enzyme known as *C3 convertase*. The other C2 fragments have kinin-like activity and increase vascular permeability. $\overline{C42}$ splits C3 into fragments, one of which (C3a) has chemotactic and anaphylatoxic properties. C3b joins the cascade. $\overline{C1423}$ acts on C5, splitting it into the smaller C5a (anaphylatoxic and chemotactic) and the larger C5b, which joins the cascade. C6 and C7 then join. A heat stable trimolecular complex C567 is formed, some of which binds to the cell membrane and prepares it for lysis by C8 and C9, which join the reaction subsequently. Most of C567 escapes and serves to amplify the reaction by absorbing to unsensitised 'bystander' cells and rendering them susceptible to lysis by C8 and C9. The C567 complex which remains unbound has chemotactic and leucocyte activating properties. The mechanism of lysis is by the production of 'holes' approximately 100Å in diameter on the cell membrane. This disrupts the osmotic integrity of the membrane, leading to the release of the contents of the cell.

Alternative pathway of C Pillemer

The central process in the complement cascade is the activation of C3, which is the major component of C. In the classical pathway, activation of C3 is achieved by the C3 convertase ($\overline{C42}$). The activation of C3, without the prior participation of C142, is known as the 'alternative pathway'.

The first example of such alternative pathways was the demonstration by Pillemer (1954) of the 'properdin system' as a group of serum proteins contributing to antimicrobial defence without

requiring specific antibodies. The normal serum protein properdin reacts with 'zymosan' (a polysaccharide from yeast cell wall) in the presence of Mg^{++} to form a PZ complex which activates C3 directly, without affecting C1, C4 and C2. The properdin pathway also requires the participation of other serum factors belonging to the 'properdin system' such as Factor A (which has been identified as C3) and Factors B, D, I and H.

Another example is the anticomplementary activity of cobra venom, which, along with Factor B present in serum, acts directly on C3. It has been found that some immunoglobulins that do not fix C (IgA, and possibly IgE) are capable of activating the alternative pathway. Some of the interactions between C and other biologically active systems of the blood (such as coagulation, fibrinolytic and kinin systems) may be mediated by the alternative pathway.

Activation of the C system can be triggered in many other ways. The following are examples:

1. C activated at a site by antigen-antibody

TABLE 14.1

The Complement Cascade: Classical Pathway

Components	Reaction sequence	Biological activity of released fragments
	E + A	
C1 (Ca^{++}; q,r,s)	EA	
C4	EAC1	C4a, C4b
C2 (Mg^{++})	EAC $\overline{14b}$	C2b, C2a, C2 kinins increases vascular permeability
C3	EAC $\overline{14b2a}$	C3a anaphylatoxin, chemotactic
C5,6,7	EAC $\overline{14b2a3b}$	Initiates membrane damage; C5a anaphylatoxin, chemotactic; C567 sensitisation of bystander cells
C8,9	EAC $\overline{14b2a3b5b67}$	Prepares cell for lysis, increases susceptibility to killer lymphocytes.
	EAC $\overline{1-9}$	Cell lysis

complexes may 'spread' to other sites (contagion of C activation). The activated C1 ~~may spread~~ from antibody to antibody. Another mechanism is the ability of C567 to render neighbouring 'bystander' cells responsive to lysis by C8 and C9, a process known as 'reactive lysis'.

2. Aggregated antibody molecules, even in the absence of antigen, ~~can behave as~~ antigen-antibody complexes and initiate C activity. Such aggregation may occur spontaneously at high immunoglobulin concentrations or may be induced by heating or treatment with a variety of chemicals (e.g., tannic acid). Particulate materials, such as kaolin and latex, can aggregate immunoglobulin on their surfaces. Uric acid crystals in gouty lesions may also cause such aggregation of immunoglobulins.

3. Certain substances other than immunoglobulins can activate C. These include DNA, polyinosinic acid and bacterial endotoxins. Enzymatic activation of C1 can be effected by plasmin and trypsin

Regulation of C activation

The generally evanescent nature of the enzymes participating in the complement cascade acts as a built-in control mechanism preventing prolonged C activity.

Normal serum contains natural inhibitors of C which provide a homeostatic mechanism for the regulation of C activity. A heat labile alpha neuraminoglycoprotein in serum inhibits C1 esterase as well as a number of other esterases found in plasma, such as plasmin, kininogen and activated Hageman factor. While this C1 inhibitor does not prevent the normal progress of the complement cascade, it exercises a check on its autocatalytic prolongation. Deficiency of the C1 inhibitor is associated with hereditary angiooedema.

Another inhibitor prevents the prolonged action of C3 convertase. A beta globulin called 'conglutinogen activating factor' (KAF) provides a homeostatic control of C3 activation, particularly by the alternative pathway. The genetic absence of KAF leads to an immune deficiency state associated with exhaustion of C by the alternative pathway.

Human serum also contains an enzyme, the anaphylatoxin inactivator which destroys the biologic activities of C3a, C4a and C5a. Many other inhibitors of activated complement components have been described, but not adequately characterised.

Biological effects of C

Complement mediates immunological membrane damage (cytolysis, bacteriolysis), amplifies *the inflammatory response and participates in the* pathogenesis of certain hypersensitivity reactions. It exhibits antiviral activity and promotes phagocytosis and immune adherence. It also interacts with coagulation, fibrinolytic and kininogenic systems of blood

The classical C pathway results in bacteriolysis and cytolysis. Cells vary in their susceptibility to complement mediated lysis. Gram negative bacteria are generally sensitive to lysis while Gram positive cells are killed without lysis. The neutralisation of viruses under some conditions requires the participation of C. For example, neutralisation of herpes virus by IgM antibody requires the binding of C1, C4 and possibly C3

C fragments released during the cascade reaction help in amplifying the inflammatory response. C2 kinins are vasoactive amines and increase capillary permeability. C3a and C5a are anaphylatoxic (histamine releasing) and chemotactic. C567 is chemotactic and also brings about reactive lysis.

C participates in the cytotoxic (Type II) and immune complex (Type III) hypersensitivity reactions. The destruction of erythrocytes, following incompatible transfusion and thrombocytopenia in sedormid purpura are examples of Type II reactions. C contributes to the pathogenesis of nephrotoxic nephritis, though immunological kidney damage may occur in the absence of C also. C is required for the production of immune complex disease such as serum sickness and

Arthus reaction. Serum C components are decreased in many autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. C also plays a major role in the pathogenesis of autoimmune haemolytic anaemia, paroxysmal nocturnal haemoglobinuria and hereditary angioneurotic oedema.

During the coagulation of blood, C3 is activated, possibly by thrombin. Platelets adhere to bound C3 and undergo lysis, releasing a platelet factor (PF3) which increases the consumption of prothrombin. C6 also participates in the coagulation process, though the exact mechanism is not understood.

Endotoxin is an efficient activator of the alternative C pathway. In endotoxic shock there is massive C3 fixation and platelet adherence. Largescale platelet lysis and release of large amounts of platelet factor lead to disseminated intravascular coagulation and thrombocytopenia. Gram negative septicaemias and the Dengue haemorrhagic syndrome may have a similar pathogenesis. Depletion of C protects against the Schwartzman reaction.

C bound to antigen-antibody complexes adhere to erythrocytes or to nonprimate platelets. This reaction, called immune adherence, contributes to defence against pathogenic microorganisms as such adherent particles are rapidly phagocytosed. C3 and C4 are necessary for immune adherence.

Bovine serum contains an unusual protein called conglutinin (K) which causes clumping of particles or cells coated with C — a process known as conglutination. Conglutinin reacts exclusively with bound C3. Though conglutinin behaves as an antibody to C, it is not an immunoglobulin and requires Ca^{++} for its activity. Antibodies with conglutinin-like activity (immunoconglutinin, IK) can be produced by immunisation with complement-coated materials. They may also occur frequently in man and other mammals as autoantibodies to fixed C. The titres of serum IK rise in conditions such as infections and autoimmune diseases associated with increased fixation of C *in vivo*. High IK levels have been noticed in the saliva and jejunal secretions. They are IgA antibodies. Their significance is not known.

There have been suggestions that C may play a role in the induction of the antibody response and of immune tolerance. Information is insufficient on these aspects.

Quantitation of C and C components

Complement activity of serum is measured by

MCQ

TABLE 14.2

Clinical syndromes associated with genetic deficiencies of complement components

Group	Deficiency	Syndrome
I	C1 inhibitor	Hereditary angioneurotic oedema
II	Early components of classical pathway C1, C2, C4	S. L. E. and other collagen-vascular diseases
III	C3 and its regulatory protein C3b inactivator	Severe recurrent pyogenic infections.
IV	C5 to C8	Bacteraemia, mainly with Gram negative diplococci, toxoplasmosis
V	C9	No particular disease.

Infection & autoimmune disease

estimating the highest dilution of serum lysing sheep erythrocytes sensitised by anti-erythrocytic antibody. Estimation of individual complement components also uses haemolytic activity in a system containing excess of all complement components except the one to be measured. C components can be quantitated also by radial immunodiffusion in agar, but this method does not differentiate between active and inactive fractions.

Biosynthesis of C

M.C.Q

C components are synthesised in various sites in the body, such as intestinal epithelium (C1), macrophages (C2, C4), spleen (C5, C8) and liver (C3, C6, C9). The site of synthesis of C7 is not known. The mechanism that controls the synthesis of the C components is not clear. C is, to some extent, an 'acute phase substance', and rise in C levels (particularly C4, C3, C5 and C6) is observed during the acute phase of inflammation.

Deficiencies of the complement system

Complete or partial deficiencies of all the classical complement components and several of the C inhibitors have been described in man or animals. Some are associated with severe diseases, while in others clinical manifestations are sporadic. C deficiencies result in the host being unable to efficiently eliminate microbial antigens or circulating immune complexes. Recurrent bacterial and fungal infections and collagen diseases occur (Table 14.2).

Deficiency of the C1 inhibitor is associated with hereditary angioneurotic oedema, a condition characterised by episodic angiooedema of the subcutaneous tissues or of the mucosa of the respiratory or alimentary tracts. It may be fatal when the larynx and trachea are affected. The attack is precipitated by the local exhaustion of the reduced amount of the C1 inhibitor present, leading to the autocatalytic activation of C1 and the unrestrained breakdown of C4 and C2. The main mediator of the oedema appears to be the C2 kinin released. The attack may be treated by infusion of fresh plasma as a source of the inhibitor. Prophylactic administration of epsilon amino-caproic-acid (or its analogues) is useful. They are believed to inhibit the activation of plasma enzymes, thus sparing the small amounts of the C1 inhibitor present.

Further Reading

Spitzer, R E 1977 Immunobiology and Clinical Importance of the Complement System of Man *Advances in Paediatrics*, 24:43

Tappiener, G 1982 Disease states in genetic complement deficiency *Int J. Dermatology* 21 175

Conglutinin - Bovine serum → clumping of particle ↓ React with [illegible]

intestinal epithelium
spleen
liver &
macrophages
(FILM)

[illegible] infection &
autoimmune disease.
[illegible]

... of ... lymphoid organs ... \ Peripheral

lymphoid cells — Lym., Pl. cells (sp. immu.)

Reticulo endo. — Phagocytic cells (non. sp. immu.) scavenger function

Immu. response < CMI by sensitised lymphocytes
AMI by Ab. produced by plasma cells.

15 Structure and Functions of the Immune System

The lymphoreticular system is a complex organisation of cells of diverse morphology distributed widely in different organs and tissues of the body. This system is responsbile for specific immunity. Lymphoreticular cells consist of lymphoid and reticuloendothelial components, with clearly demarcated functions. The ~~lymphoid cells — lymphocytes and plasma cells~~ — are primarily ~~concerned with the specific immune response~~. The ~~phagocytic cells~~ forming part of the ~~reticuloendothelial system~~, are ~~primarily concerned with the scavenger functions of eliminating effete cells and foreign~~ particles. They contribute to ~~nonspecific immunity by~~ removing microorganisms from blood and tissues. They also play a role in specific immunity, both in the afferent and in the efferent limbs of the immune response.

The functional anatomy of the lymphoid system can be appreciated only against the background of the 'two component concept' of immunity. The immune response to an antigen, whatever be its nature, can be of two broad types — the humoral or antibody mediated immunity (AMI) and the cellular or cell mediated immunity (CMI). Humoral immunity is mediated by antibodies produced by plasma cells, while cellular immunity is mediated directly by sensitised lymphocytes. Cells for each of these components develop through separate channels and remain independent, though they may also interact in some instances (Fig. 15.1).

The lymphoid system consists of the lymphoid cells (lymphocytes and plasma cells) and lymphoid organs. Based on the different roles they perform, lymphoid organs can be classified into the central (primary) and the peripheral lymphoid organs. The central lymphoid organs are lymphoepithelial structures in which the precursor lymphocytes proliferate, develop and acquire immunological capability. The thymus and the bursa of Fabricius, in birds, are primary lymphoid organs, being responsible for the cellular and humoral immune responses, respectively. The equivalent of the avian bursa in mammals is the bone marrow. After acquiring immunocompetence, the lymphocytes migrate along blood and lymph streams, accumulate in the peripheral lymphoid organs, and following antigenic stimulus, effect the appropriate immune response. The spleen and lymph nodes constitute the major peripheral lymphoid organs. Lymphoid tissues in the gut, lungs, liver and bone marrow and lymphoid collections in the adventitious tissue of all organs also form part of the peripheral lymphoid system.

Central lymphoid organs

Thymus: The thymus anlage develops from the epithelium of the third and fourth pharyngeal pouches at about the 6th week of gestation and by the 8th week, grows into a compact epithelial structure. Mesenchymal stem cells (precursors of lymphocytes) from the yolk sac, fetal liver and bone marrow reach the thymus and differentiate

into the thymic lymphoid cells (thymocytes). The thymus acquires its characteristic lymphoid appearance by the 3rd month of gestation. It is thus the first organ in all animal species to become predominantly lymphoid. In man, the thymus reaches its maximal relative size just prior to hirth. It continues to grow till about the 12th year. After puberty, it undergoes spontaneous progressive involution, indicating that it functions best in early life.

The thymus is located behind the upper part of the sternum. Aberrant thymic tissues are often found in neighbouring sites. It has two lobes surrounded by a fibrous capsule. Septa arising from the capsule divide the gland into lobules which are differentiated into an outer cortex and an inner medulla. The cortex is crowded with actively proliferating small lymphocytes The medulla consists mainly of epithelial cells and scattered lymphocytes amidst which are the Hassall's corpuscles, whorl-like aggregations of epithelial cells.

Till recently, the thymus was an organ without any recognised function. The fortuitous observations by Good (1954), of thymoma and impaired immunity in a patient, and by Miller (1961), of immunodeficiency in neonatally thymectomised mice, paved the way for the understanding of the pivotal role of the organ in the development of cell mediated immunity. The primary function of the thymus is the production of thymic lymphocytes. It is the major site for lymphocyte proliferation in the body. But, of the lymphocytes produced, only about one per cent leave the thymus. The rest are destroyed locally. The reason for this apparently wasteful process is not known. In the thymus, the lymphocytes acquire new surface antigens (Thy antigens). Lymphocytes produced in the thymus are called 'thymus (T dependent) lymphocytes' or 'T cells'. Unlike lymphocyte proliferation in the peripheral organs, in the thymus it is not dependent on antigenic stimulation. In fact, peripheral antigenic stimuli do not lead to any immune response in the thymus. Antigen introduced directly into the thymus may lead to a local immune response.

The thymus confers immunological competence on the lymphocytes during their stay in the organ Prethymic lymphocytes are not immunocompetent In the thymus they are 'educated' so that they become capable of mounting cell mediated immune response against appropriate anti-

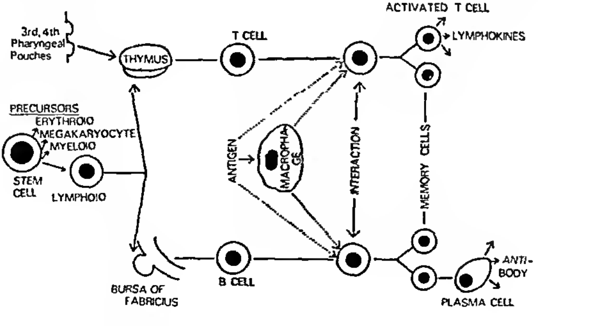

Fig 15 1 Development of T and B cell systems

Peyer's patches or scattered isolated lymphoid follicles — collectively called the mucosa-associated lymphoid tissues (MALT). Such lymphoid tissues in the gut, from the adenoids and tonsils to the follicles in the colon are called the gut-associated lymphoid tissue (GALT) and those in the respiratory tract, the bronchus associated lymphoid tissue. (BALT)

MALT contain lymphoid as well as phagocytic cells. Both B and T cells are present. While the predominant immunoglobulin produced in the mucosa is secretory IgA, other immunoglobulin classes, IgG, IgM and IgE are also formed locally.

There appears to be a free traffic of antigen specific effector lymphocytes between the various mucosal and secretory areas, so that an antigenic exposure at one site may cause production of the specific antibody at the other mucosal and secretory sites also. This indicates the existence of a common *mucosal or secretory immune system* and explains the superiority of oral or nasal immunisation over the parenteral route for many enteric and respiratory infections.

Cells of the lymphoreticular system

The cells of the lymphoreticular system consist of structural cells (reticulum cells, endothelial cells and fibroblasts) and those that subserve specialised immunological functions (lymphocytes, plasma cells and macrophages). Their classification into one or other category may not always be easy, as they form a spectrum of cells with characters of one merging into those of others. Such a classification may also be artifical as cells of the same morphology may not be functionally identical. Moreover, at least some of them can trans-

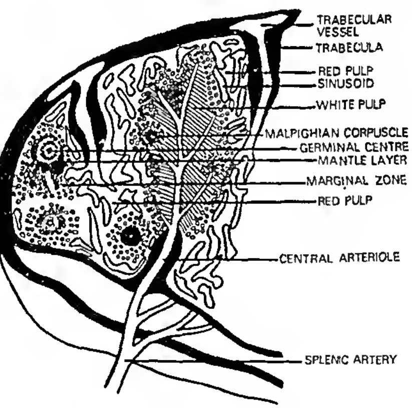

Fig. 15.3 Schematic diagram of splenic architecture

form from one to another depending on the functional need of the moment.

Lymphocytes: Lymphocytes are small, round cells found in peripheral blood, lymph, lymphoid organs and in many other tissues. In peripheral blood, they constitute 20–45 per cent of the leucocyte population, while in lymph and lymphoid organs they form the predominant cell type. Ehrlich (1879) who introduced a staining technique for blood cells described lymphocytes as nonmotile end cells with no recognisable function. Lymphocytes are now recognised to be the major cellular elements responsible for immunological responses.

According to their size, lymphocytes can be classified into small (5–8 μ), medium (8–12 μ) and large (12–15μ) lymphocytes. The small lymphocytes are the most numerous. They consist of a spherical nucleus with prominent nuclear chromatin and a thin rim of cytoplasm, containing scattered ribosomes, but virtually devoid of endoplasmic reticulum or other organelles. They are capable of slow motility, and during movement assume a 'hand mirror' form, with the nucleus in front and the cytoplasm as a tail behind.

Depending on their life span, they can be classified as short-lived and long-lived lymphocytes In man, the short-lived lymphocytes have a life span of about two weeks, while the long lived cells may last for three years or more, or even for life. The short-lived lymphocytes are the effector cells in immune response, while the long-lived cells act as the storehouse for immunological memory. The long-lived cells are mainly thymus derived.

Lymphopoiesis takes place in at least three sites — the bone marrow, central lymphoid organs and the peripheral lymphoid tissues. These populations of lymphocytes do not remain distinct, but mix together in the process known as 'lymphocyte recirculation'. There is a constant traffic of lymphocytes through the blood, lymph, lymphatic organs and tissues. This recirculation ensures that, following introduction of antigen into any part of the body, lymphocytes of appropriate specificity would reach the site during their ceaseless wandering and mount an immune response. Recirculating lymphocytes can be recruited by the lymphoid tissues whenever necessary. Recirculating lymphocytes are mainly T cells. B cells tend to be more sessile. Chronic thoracic duct drainage will therefore result in selective T cell depletion.

A lymphocyte that has been 'educated' by the central lymphoid organs becomes an 'immunologically competent cell' (ICC) Such cells, though not actually engaged in an immunological response, are nevertheless fully qualified to undertake such a responsibility when appropriately stimulated by an antigen. They subserve the following functions — recognition of antigens, storage of immunological memory, and immune response to specific antigens. Lymphocytes have antigen recognition mechanisms on their surface, enabling each cell to recognise only one antigen, or probably a small number of antigens. The reaction of an immunocompetent cell to its specific antigen may be induction of either 'tolerance' or of immune response. The nature of immune response depends on whether the lymphocyte is a B or T cell. Stimulated T cells produce certain activation products (*lymphokines*) and induce CMI, while stimulated B cells divide and transform into plasma cells which synthesise immunoglobulins.

Circulating lymphocytes can be classified as T and B cells based on the following features:

1. T cells bind to sheep erythrocytes at 37°C forming rosettes (SRBC or E rosette) while B cells do not.
2. B cells bind to sheep erythrocytes coated with antibody and complement forming EAC rosettes, T cells do not. EAC rosette formation is due to the presence of a C3 receptor on B cell surface.
3. T cells have thymus specific antigens on their surface, which are absent on B cells.
4. B cells have immunoglobulins on their surface. They also possess surface receptors for the *Fc* fragment of IgG. They are absent on T cells, which have on their surface a homolog-

ous determinant called Ti which is the antigen recognition unit.

5. T cells undergo blast transformation, evidenced by enhanced DNA synthesis, on treatment with mitogens such as phytohaemagglutinin (PHA) or Concanavalin A (Con A), while B cells undergo similar transformation with bacterial endotoxins.
6. Viewed under the scanning microscope, T cells are generally free of cytoplasmic surface projections, while B cells have an extensively filamentous surface, with numerous microvilli (Table 15.1).

T cells can be divided into different *subpopulations* or *subsets* based on their functional capacity and on their surface antigenic determinants.

Functionally, T cells may be classified as *regulator* cells and *effector* cells. Regulator T cells may be *helper (inducer)* cells or *suppressor* cells. Helper T cells facilitate B cell response to many antigens. Suppressor T cells inhibit antibody production by B cells and immune reactions by effector T cells. Immune response is regulated by mutually opposing influences of helper and suppressor T cells. Their balanced activity produces optimal immune response. Overactivity of helper cells or decreased suppressor cell activity causes abnormal immune responses as seen in autoimmunity. Diminished helper cell function or increased suppressor cell activity leads to immunodeficiency. Helper cells constitute about 65 per cent and suppressor cells about 35 per cent of circulating T lymphocytes.

Effector cells may be cytotoxic cells, those mediating delayed type hypersensitivity or those which undergo rapid proliferation in mixed lymphocyte reactivity (Table 15.2).

T lymphocytes can also be differentiated into various subsets based on antigenic determinants present on their surface. Their surface antigens change progressively during the course of their maturation in the thymus and also with their functional differentiation. Monoclonal antibodies are used to identify the antigenic subsets of T cells.

The earliest lymphoid cells within the thymus, constituting about 10 per cent of thymic lymphocytes show T11 antigen which is the sheep erythrocyte (E or SRBC) rosette receptor present in all T cells. They also carry T9 and T10 which are not exclusive T cell antigens, being present also on many bone marrow cells. With maturation, they lose T9 and acquire T6, T4 and T8 antigens. About 70 per cent of thymic lymphocytes are of this stage. On further maturation, they lose T6, acquire T1, T3 and T12 and segregate into the reciprocal T4 and T8 subsets. T4 is specific for helper/inducer and T8 for suppressor/cytotoxic

TABLES 15.1

Some distinguishing characteristics of T cells, B cells and macrophages

Property	T cell	B cell	Macrophage
Surface immunoglobulins	−	+	−
Receptor for *Fc* piece of IgG	−	+	−
EAC rosette (C3 receptor)	−	+	−
SRBC rosette	+	−	−
Thymus specific antigens	+	−	−
Numerous microvilli on surface	−	+	−
Blast transformation with:			
a. PHA	+	−	−
b. Concanavalin A	+	−	−
c. Endotoxins	−	+	−
Phagocytic action	−	−	+
Adherence to glass surface	−	−	+

TABLE 15.2
T lymphocyte subsets

A.	Regulatory T cells.
	1) Helper cells (T_H)
	2) Suppressor cells (T_s)
B.	Effector cells.
	1) Cytotoxic T lymphocytes (CTL)
	2) Delayed type hypersensitivity (DTH)
	3) *Mixed lymphocyte reactivity* (MLR)

subsets. At this stage immunocompetence is acquired, but is not fully developed till the T cells are exported from the thymus. Mature T cells also possess the Ti receptor which is the antigen recognition unit of the T cells, homologous to the surface immunoglobulin on B lymphocytes. Ti resembles immunoglobulin structurally as it consists of two subunits composed of variable and constant regions. Ti is linked to T3 on T cell surface (Fig. 15.4).

The above antigens on the T cell surface, detected by monoclonal antibodies, are expressed on molecules that play an important role in differentiation and function of the cells. These molecules are referred to as 'clusters of differentiation' (CD) and have been designated by numbers. The old terminology and the corresponding new CD designations of the important T cell surface molecules are given below:

Old designation of T cell surface antigen	Present designation of CD molecule
T_{11}	CD_2
T_3	CD_3
T_4	CD_4
T_8	CD_8

During maturation and differentiation in the thymus, T cells also learn to recognise self-MHC antigens. T4 (CD4) cells recognise Class II MHC antigens and T8 (CD8) cells recognise MHC class I antigens.

B cells also exist in different subpopulations — those that selectively form the immunoglobulin classes M, G, A, D and E.

Of the lymphocytes in peripheral circulation, about 55–75 per cent are T cells and about 15–30 per cent B cells. The small proportion of lymphocytes which cannot be classified are known as *null cells*. Their nature and function are not fully understood.

Among the null cells, a distinct subpopulation of cytotoxic cells has been recognised, which possess surface receptors for the *Fc* part of IgG. They are capable of lysing or killing target cells sensitised with IgG antibodies. They are known as *killer (K) cells*. K cells do not require complement. They are responsible for antibody-dependent cell mediated cytotoxicity (ADCC), in contrast to the action of cytotoxic T lymphocytes which is independent of antibody.

Another subpopulation of null cells is the *natural killer* (NK) cells. These are large lymphocytes containing azurophilic granules in the cytoplasm and have therefore been called *large granular lymphocytes* (LGL). NK cells are capable of nonspecific killing in virally transformed target cells and are involved in allograft and tumour rejection. They differ from K cells in being independent of antibody. Gamma interferon increases NK cell activity. Their cytotoxic activity is nonspecific and can be demonstrated on tumour cell lines or foetal fibroblast cultures. NK cells are present in the spleen and peripheral blood in man and animals. Though their exact functional significance is not known, they are believed to play an important role in antiviral and antitumour immunity.

Plasma cells: Antigenically stimulated B cells undergo blast transformation, becoming successively plasmablasts, intermediate transitional cells and plasma cells. The mature plasma cell is the antibody secreting cell. It is an oval cell, about twice the size of a small lymphocyte, with an eccentrically placed oval nucleus, containing large blocks of chromatin located peripherally (cartwheel or clockface appearance). The cytoplasm is large and contains abundant endoplasmic reticulum and a well developed Golgi apparatus. It is structually designed to be an immunoglobu-

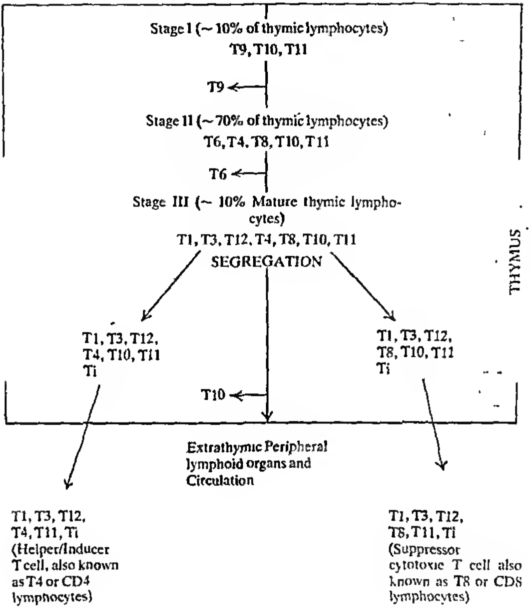

Fig 15 4 Surface antigens on T lymphocytes during their stages of development and differentiation

lin producing factory. Plasma cells are end cells and have a short lifespan of two or three days. A plasma cell makes an antibody of a single specificity, of a single immunoglobulin class and allotype, and of a single light chain type only. An exception is seen in the primary antibody response, when a plasma cell producing IgM initially, may later be switched on to IgG production.

While plasma cell is the antibody producing cell *par excellence*, lymphocytes, lymphoblasts and transitional cells may also synthesise immunoglobulins to some extent.

Phagocytic cells: Phagocytosis is phylogenetically the oldest defence mechanisms in animals. Originating in protozoa as a combined mechanism for nutrition and defence, the phagocyte lost its trophic functions with the development of digestive enzymes along the course of evolution, and in higher organisms it specialised in the removal of foreign and autochthonous particles. The importance of phagocytosis in defence has been known since its discovery by Metchnikoff (1882). Its vital role in preventing infections has been emphasised by the recent discovery of a congenital deficiency of phagocytosis ('chronic granulomatous disease'), in which the patient succumbs to recurrent bacterial infections, even though T and B cell functions remain normal. The phagocytic cells of the body are the mononuclear macrophages (of blood and tissues) and the polymorphonuclear microphages.

The blood macrophages (monocytes) are the largest of the lymphoid cells found in peripheral blood (12–15μ). The tissue macrophages (histiocytes) are larger (15–20μ). Mononuclear macrophage cells originate in the bone marrow from precursor cells and become monocytes in about six days. Monocytes in circulation have an approximate half life of three days. They leave the circulation and reach various tissues to become transformed to macrophages, with morphological and functional features characteristic of the tissues, such as alveolar macrophages in lungs and Kupffer cells in liver. Tissue macrophages proliferate locally and survive for months. Multinucleated cells and epitheloid cells seen in granulomatous inflammatory lesions such as tuberculosis originate from mononuclear macrophage cells.

The primary function of macrophage is phagocytosis. These cells move slowly in a ponderous-and-purposeful-manner, their abundant cytoplasm thrusting out restless pseudopodia that glide harmlessly past normal body cells, but engulf effete cells and foreign particles. They accumulate in areas of inflammation or of tissue damage by chemotaxis. Particles sensitised by antibodies are phagocytosed more readily. The phagocytosed particle is held inside a vacuole (phagosome), the membrane of which fuses with a lysosome, forming a 'phagolysosome'. Lysosomal enzymes digest the particle, the remnants being extruded from the cell. While phagocytosis is an effective defence against most microorganisms, some (such as the bacilli of typhoid, brucellosis and tuberculosis) resist digestion and may multiply in the cells and be transported in them to other locations.

The macrophage may participate in several ways in the induction and execution of the specific immune response. It traps the antigen and provides it, in optimal concentration to the lymphocytes. Too high a concentration of antigen may be tolerogenic, and too low a concentration may not be immunogenic. It has also been shown that with some antigens, prior processing by macrophages is an essential prerequisite for induction of antibodies.

The processing and presentation of antigen by the macrophage to T cells require that both the cells possess surface determinants coded for by the same major histocompatability complex (MHC) genes. The T cell can accept the processed antigen only if it is presented by macrophage carrying on its surface the self-MHC determinant — the so called immune associated or *Ia* antigen. When the macrophage bears a different *Ia* antigen, it cannot cooperate with the T cell. This is known as *MHC restriction*.

The functional efficiency of macrophage can be increased in many ways. They may be 'activated' by lymphokines, complement components or interferon. Activated macrophages are not antigen specific. For example, activated macrophages from animals infected with one microorganism are cytotoxic to tumour cells as well as to many other microorganisms. Activated macrophages show morphological and functional changes as compared to unstimulated quiescent macrophages. They are larger, adhere better and spread faster on glass and are more phagocytic.

They secrete a number of biologically active substances, including interleukin-1 (formerly called leukocyte activating factor), which acts as endogenous pyrogen and also induces synthesis of interleukin-2 by T cells. Interleukin-2 facilitates activation of T cells. When stimulated by cytophilic antibodies and certain lymphokines, macrophages become 'armed' Such armed macrophages are capable of antigen specific cytotoxicity, which is important in antitumour activity and graft rejection.

The microphages are the polymorphonuclear leucocytes of blood — neutrophils, eosinophils and basophils. Neutrophils are actively phagocytic and form the predominant cell type in acute inflammation. The phagocytic property of neutrophils is nonspecific, except for its augmentation by opsonins. They do not appear to have any role in specific immune processes. Eosinophilic leucocytes are found in large numbers in allergic inflammation, parasitic infections and around antigen-antibody complexes, but their function is not clear. They are primarily inhabitants of tissues rather than of the bloodstream. The distinctive feature of the eosinophil is the presence of two types of granules — small, round, homogeneous and large ovoid. The granules contain a variety of hydrolytic enzymes which may in some manner contribute to the manifestations of hypersensitive reactions. Eosinophils possess phagocytic activity but only to a limited degree.

Basophil leucocytes are found in blood and tissues (mast cells). Their cytoplasm has large numbers of prominent basophilic granules containing heparin, histamine, serotonin and other hydrolytic enzymes. Degranulation of mast cells, with release of pharmacologically active agents constitutes the effector mechanism in anaphylactic and atopic allergy.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

The primary function of the immune system is the recognition and elimination of foreign cells and antigens that enter the body. Tissues and organs grafted from one individual to another member of the same species (allografts) are recognised as foreign and rejected. It was the early work of Gorer in the 1930s into the antigens responsible for allograft rejection in inbred mice that led to the discovery of the major histocompatibility complex (MHC).

Gorer identified two blood group antigen systems in mice, one of which (antigen 1) was common to all strains. Antigen 2 was found in some strains only and appeared to be responsible for allograft rejection. This was called the H-2 antigen (H for histocompatibility). By *histocompatibility antigen* is meant cell surface antigens that induce an immune response leading to rejection of allografts (See Chapter 20). The H-2 antigen system was found to be the *major histocompatibility antigen* for mice and coded for by a closely linked multi-allelic cluster of genes, which was called the *major histocompatibility complex*. The development of congenic and recombinant strains of mice by Snell enabled the detailed analysis of various loci of this complex. For their work on MHC and genetic control of immune response, Snell, Dausset and Benacerraf were awarded the Nobel Prize for Medicine in 1980.

The HLA complex: The major antigens determining histocompatibility in man are alloantigens found characteristically on the surface of leucocytes. They have been termed *human leucocyte antigens* (HLA) and the set of genes coding for them the *HLA complex*.

The HLA complex of genes is located on the short arm of chromosome 6 (Fig 15.5). It consists of seven genetic loci grouped into two classes, — HLA-A, -B and -C constituting Class I, HLA-D, -DR, -DQ and -DP constituting Class II. The gene products (antigens) of Class I and Class II loci are called Class I and Class II antigens, respectively. (A locus is the position on the chromosome where a particular gene is located.) The HLA complex also bears a *complement region*, which has been mapped between the HLA-B and HLA -DR loci. Located here are genes coding for the second and fourth complement components (C2 and C4) of the classical

complement pathway and the properdin factor B (BF) of the alternative pathway.

HLA loci are multiallelic, i.e., the gene occupying the locus can be any one of several alternative forms (alleles). As each allele determines a distinct product (antigen), the HLA system is very pleomorphic. For example, at least 23 distinct alleles have been identified at HLA locus A and 47 at B.

The nomenclature of the HLA system is regulated by a special committee under the auspicies of the World Health Organisation. The same designation is used for an HLA allele and its product. Officially recognised alleles and their corresponding antigens are designated by the locus and a number (e.g., HLA - A1, HLA - B5, HLA-DR 7, etc). Provisional alleles and their antigens awaiting official recognition by the Committee have a w (for 'workshop'), the w being deleted once formal recognition is granted.

HLA antigens are two-chain glycoprotein molecules anchored on the surface membrane of cells. Class I antigens (A,B and C) are found on the surface of virtually all cells. They are the principal antigens involved in graft rejection and cell mediated cytolysis. HLA Class II antigens have a more restricted distribution, being found principally on macrophages, activated T lymphocytes, and particularly on B lymphocytes. HLA-D antigens are primarily responsible for the graft-verses-host response and the mixed leucocyte reaction (MLR). The Immune response (*Ir*) genes which control the immunological responses to specific antigens are believed to be situated in the HLA Class II region. While *Ir* genes have been studied extensively in mice and located in the *I* region of mouse MHC, their exact localisation and significance in man have not been clarified. It has been suggested that they may be associated with HLA-DR locus. The antigens coded for by the *Ir* genes are called the *Ia* (*I* region associated) antigens.

HLA typing: Antisera for HLA typing are obtained principally from multiparous women as they tend to have antibodies to the HLA antigens of their husbands, due to sensitisation during pregnancy. Monoclonal antibodies to HLA antigens are being developed. Typing is done serologically by microcytotoxicity, which tests for complement mediated lysis of peripheral blood lymphocytes by a standard set of typing sera. However, serological typing is not possible for HLA-D and HLA-DP antigens, which are detected by mixed leucocytic reaction (MLR) and primed lymphocyte typing (PLT), respectively.

The HLA antigens coded for by the combination of alleles at each locus on one strand of a chromosome pair represent the *haplotype*. The complete HLA type of an individual comprises the antigens represented on both strands of the diploid chromosome and so will consist of two haplotypes (e.g., HLA-A1, -A2; -B7, -B12; -Cw3, - Cw8; Dw4, -Dw7; -DR1, -DR7; DQw1, -DQw3; -DPw4, -DPw6).

Due to the extreme pleomorphism of the HLA system, delineation of the HLA type provides a method of typing of individuals, which is far more discriminating than blood grouping. HLA typing is used primarily for testing compatibility between recipients and potential donors before tissue transplantation. It has applications also in disputed paternity. As the prevalence of HLA types vary widely among different human races and ethnic groups, HLA typing is used in anthropological studies. An association has been observed between HLA types and certain diseases. Such diseases are generally of uncertain

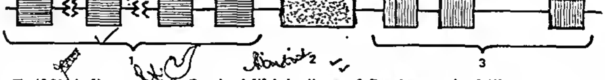

Fig. 15.5 Major Histocompatibility Complex. 1. HLA class II region 2. Complement region. 3. HLA Class I region

aetiology, associated with immunological abnormalities and with a hereditary tendency. For example, strong association has been found between ankylosing spondylitis and HLA-B27, rheumatoid arthritis and HLA-DR4, and many autoimmune conditions and HLA-DR3.

MHC restriction

The importance of MHC antigen in immune reactions is indicated by the finding that T cells respond to antigens on macrophages and other accessory cells only when they are presented along with the self-MHC antigen. This is known as *MHC restriction*. Both Class I and Class II antigens operate in this phenomenon. Cytotoxic T lymphocytes from immunised mice are able to kill and lyse virus infected target cells only when T cells and target cells are of the same MHC type, so that the T cells can recognise Class I MHC antigens on the target cells. Helper T cells can accept antigen presented by macrophages only when the macrophage bears the same Class II MHC molecules on the surface. For T cells participating in delayed type hepersensitivity the antigen has to be presented along with Class II MHC determinants. MHC restriction has been demonstrated in man also.

Further Reading

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16 Immune Response

The specific reactivity induced in a host by an antigenic stimulus is known as the immune response. In infectious disease it is generally equated with protection against invading microorganisms. But the immune response has a much wider scope and includes reactions against any antigen, living or nonliving. It may lead to consequences that are beneficial, indifferent or injurious to the host. It also includes the state of specific nonreactivity (tolerance) induced by certain types of antigenic stimuli. The immune response can be of two types—the humoral (antibody mediated) and the cellular (cell mediated) types. The two are usually developed together, though at times one or the other may be the predominant or exclusive type. They usually act in conjunction, but may sometimes act in opposition.

Antibody mediated immunity (AMI) provides primary defence against most extracellular bacterial pathogens, helps in defence against viruses that infect through the respiratory or intestinal tracts, prevents recurrence of virus infections and participates in the pathogenesis of immediate (types 1,2 and 3) hypersensitivity and certain autoimmune diseases. Cell mediated immunity (CMI) protects against fungi, viruses and facultative intracellular bacterial pathogens, participates in rejection of homografts and graft-versus-host reaction, provides immunological surveillance and immunity against cancer, and mediates the pathogenesis of delayed (type 4) hypersensitivity and certain autoimmune diseases.

HUMORAL IMMUNE RESPONSE

The production of antibodies consists of three steps:

1. the entry of the antigen, its distribution and fate in the tissues and its contact with appropriate immunocompetent cells (*the afferent limb*),
2. the processing of antigen by cells and the control of the antibody forming process (*central functions*), and
3. the secretion of antibody, its distribution in tissues and body fluids and the manifestations of its effects (*efferent limb*).

Antibody production follows a characteristic pattern consisting of: 1) a *lag phase*, the immediate stage following antigenic stimulus during which no antibody is detectable in circulation, 2) a *log phase* in which there is steady rise in titre of antibodies, 3) a *plateau* or steady state where there is an equilibrium between antibody synthesis and catabolism, and 4) the *phase of decline* during which catabolism exceeds production and the titre falls (Fig. 16.1).

Primary and secondary responses

The antibody response to an initial antigenic stimulus differs qualitatively and quantitatively from the response to subsequent stimuli with the same antigen. The former is called the 'primary response' and the latter the 'secondary response' (Fig. 16.2). The primary response is slow, sluggish and shortlived, with a long lag phase and low titre of antibodies that do not persist for long. In contrast, the secondary response is prompt, powerful and prolonged, with a short or negligible lag phase and a much higher level of antibodies that lasts for long periods. The antibody formed in the primary response is predominantly IgM and in the secondary response IgG. The early antibody

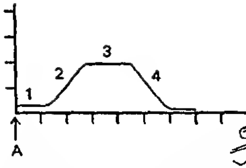

Fig 16 1 Primary immune response A antigenic stimulus 1 latent period 2 rise in titre of serum antibody 3. steady state of antibody titre 4 decline of antibody titre

is more specific but less avid than the late antibody. The duration of the lag phase and the persistence of the antibody vary with the nature of the antigen. With some antigens such as diphtheria toxoid, the lag phase in the primary response may be as long as 2–3 weeks, while with pneumococcal polysaccharide, antibodies can be detected as early as within a few hours.

A single injection of an antigen helps more in sensitising or priming the immunocompetent cells producing the particular antibody than in the actual elaboration of high levels of antibody. Effective levels of antibody are usually induced only by subsequent injections of the antigen It is

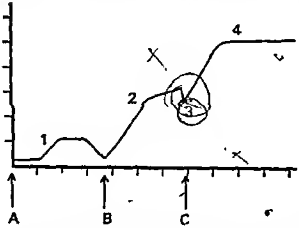

Fig 16 2 Effect of repeated antigenic stimulus A, B, C antigenic stimuli 1. primary immune response. 2. secondary immune response 3. negative phase. 4. high level of antibody following booster injection

for this reason that nonliving vaccines are given in multiple doses for active immunisation The first injection is known as the 'priming' dose and subsequent injections as 'booster' doses With live vaccines, a single dose is sufficient as multiplication of the organism in the body provides a continuing antigenic stimulus that acts both as the priming and booster doses

When an antigen is injected into an animal already carrying the specific antibody in circulation, a temporary fall in the level of circulating antibody occurs due to the combination of the antigen with the antibody. This has been called the 'negative phase'. It is followed by an increase in titre of the antibody exceeding the initial level It has been suggested that when a prophylactic vaccine is administered to a host exposed to an infection, the negative phase may actually enhance the susceptibility temporarily But this is generally not considered relevant in actual practice as it is doubtful whether the fall in circulating antibodies is associated with a true lowering of specific immunity

Fate of antigen in tissues

The manner in which an antigen is dealt with in the body depends on factors such as the physical and chemical nature of the antigen, its dose and route of entry and whether the antigenic stimulus is primary or secondary. Antigens introduced intravenously are rapidly localised in the spleen, liver, bone marrow, kidneys and lungs. They are broken down by the reticuloendothelial cells and excreted in the urine, about 70–80 per cent being thus eliminated within one or two days. In contrast, antigens introduced subcutaneously are mainly localised in the draining lymph nodes, only small amounts being found in the spleen.

Particulate antigens are removed from circulation in two phases The first is the nonimmune phase during which the antigen is engulfed by the phagocytic cells, broken down and eliminated. With the appearance of the specific antibody, the phase of immune elimination begins, during which antigen-antibody complexes are formed

and are rapidly phagocytosed, resulting in an accelerated disappearance of the antigen from circulation. With soluble antigens, three phases can be made out — equilibration, metabolism and immune elimlnation. The phase of equilibration consists of diffusion of the antigen to the extravascular spaces. During the metabolic phase, the level of antigen falls due to catabolic decay. During the phase of immune elimination, there is rapid elimination of the antigen with the formation of antigen-antibody complexes. Such complexes can cause tissue damage and may be responsible for 'immune complex diseases' such as serum sickness.

The speed of elimination of an antigen is related to the speed with which it is metabolised. Protein antigens are generally eliminated within days or weeks, whereas polysaccharides which are metabolised slowly, persist for months or years. Pneumococcal polysaccharide, for instance, may persist upto 20 years in man, following a single injection.

The presentation of antigens to immunocompetent lymphocytes occurs in two ways — by macrophages and by the dendritic cells in the lymph node follicles. With many antigens processing by macrophages appears to be a necessary prerequisite for antibody formation. Antigen processing by macrophages appears to be essential for the primary immune response to many antigens, but not for the secondary response. Antigen capture by the dendritic cells of the lymph node follicles occurs in the presence of preexisting antibody. Both macrophages and dendritic cells present the antigen, native or processed, at the cell surface, in areas of the body having a heavy traffic of lymphocytes, so that there occurs ample opportunity for the appropriate lymphocytes to recognise the antigen and initiate the immune response.

Macrophages modulate the dose of antigen presented to lymphocytes so that they are neither too low to be nonimmunogenic, nor too high to be tolerogenic. Macrophages also release soluble factors such as interleukin-1 which help in the initiation of the immune response.

Production of antibodies

The cells that recognise antigens and initiate antibody synthesis are the immunocompetent B lymphocytes that carry surface receptors which are IgM or IgG in nature. The surface receptors are specific for individual antigenic determinants. The receptors on a single antigen reactive cell are directed towards a single determinant group. When an antigen reactive cell is stimulated by contact with the appropriate antigenic determinant, it undergoes clonal proliferation and blast transformation, becoming converted into plasma cells which are end cells that synthesise and secrete antibodies. All antibody molecules synthesised by a single clone will be uniform with regard to antibody specificity, light and heavy chain markers and allotype. An exception is shown by cells that initially secrete IgM, later switching over to form IgG, IgA or IgE.

While B lymphocytes are the cells responsible for antibody production, humoral response to many antigens requires the participation of T lymphocytes. These antigens called *thymus-dependent antigens*, include erythrocytes, serum proteins and a variety of protein-hapten conjugates. Some antigens such as polymerised flagellin, ferritin and several polysaccharides do not require T cell participation. A characteristic feature of these *thymus-independent antigens*, is that they consist of repeating surface units or epitopes. The antibody induced by them is largely of the IgM class, associated with little or no immunological memory.

Antibody formation by B cells is subject to the regulatory effect of T cells. Helper T cells (T_H) stimulate and suppressor T cells (T_S) inhibit antibody formation. Optimal antibody response depends on the balanced activity of T_H and T_S cells.

Following antigenic stimulus, not all B cells get converted into plasma cells. A small proportion of them develop into 'memory cells' which have a long lifespan and serve to recognise the same antigen when introduced subsequently. The increased antibody response during secondary

antigenic stimulation is due to the presence of memory cells induced by the primary contact with the antigen.

Monoclonal antibodies

A single antibody forming cell or clone produces antibodies specifically directed against a single antigen or antigenic determinant only. When a clone of lymphocytes or plasma cells undergoes selective proliferation, as in multiple myeloma, antibodies with a single antigenic specificity accumulate. Such antibodies produced by a single clone and directed against a single antigenic determinant are called *monoclonal antibodies*. Monoclonal antibodies are very useful tools for diagnostic and research techniques.

An ingenious method for largescale production of monoclonal antibodies against any desired antigen was described by Kohler and Milstein in 1975. In recognition of the great importance of this hybridoma technology, the Nobel Prize for Medicine was awarded to them in 1984. Hybridomas are somatic cell hybrids produced by fusing antibody forming spleen cells with myeloma cells. The resultant hybrid retains the antibody producing capacity of the spleen cell and the ability of myeloma cells to multiply indefinitely (Fig. 16.3). Lymphocytes from the spleen of mice immunised with the desired antigen are fused with mouse myeloma cells grown in culture and deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT). The fused cells are placed in a basal culture medium which does not permit growth of the enzyme deficient myeloma cells. As normal lymphocytes cannot replicate indefinitely, only hybrid cells possessing properties of both the splenic lymphocytes and myeloma cells can grow in the culture. These cultures, called *hybridomas*, are cloned and examined for production of antibodies. Clones producing antibodies against the desired antigen are selected for continuous cultivation. Such hybridomas can be maintained indefinitely in culture and will continue to form monoclonal antibodies. They may be also injected intraperitoneally in mice and monoclonal antibodies obtained by harvesting the ascitic fluid produced. Hybridoma may be frozen for prolonged storage. The discovery of hybridoma technology for production of monoclonal antibodies has created a revolution in immunology, by opening up numerous diagnostic, therapeutic and research applications. Monoclonal antibodies against several antigens are now available commercially.

Factors influencing antibody production

Genetic factor: The immune response is under genetic control. The differences in immune response to the same antigen shown by different individuals in a species is determined by genetic differences. The terms 'responder' and 'nonresponder' are used for individuals who are or are not capable of responding to a particular antigen. The *Ir* genes control this property.

Age: The embryo is immunologically immature. The capacity to produce antibodies starts only with the development and differentiation of lymphoid organs. The age at which embryos acquire immunological competence varies with different species. When the potential immunocompetent cell comes into contact with its specific antigen during embryonic life, the response is elimination of the cell or induction of tolerance. This is believed to be the basis for the nonantigenicity of self-antigens. During embryonic life, the developing lymphoid cells come into contact with all the tissue antigens of the body released by cellular breakdown, so that all clones of cells that have specificity towards self-antigens are eliminated.

Immunocompetence is not complete at birth, but continues to develop as the infant grows. The infant has to depend on itself for antibody production from 3–6 months of age, by which time the maternal antibodies disappear. But full competence is acquired only by about the age of 5–7 years for IgG and 10–15 years for IgA. The ontogeny of antibody response depends also on the antigens concerned. B cell responses to most

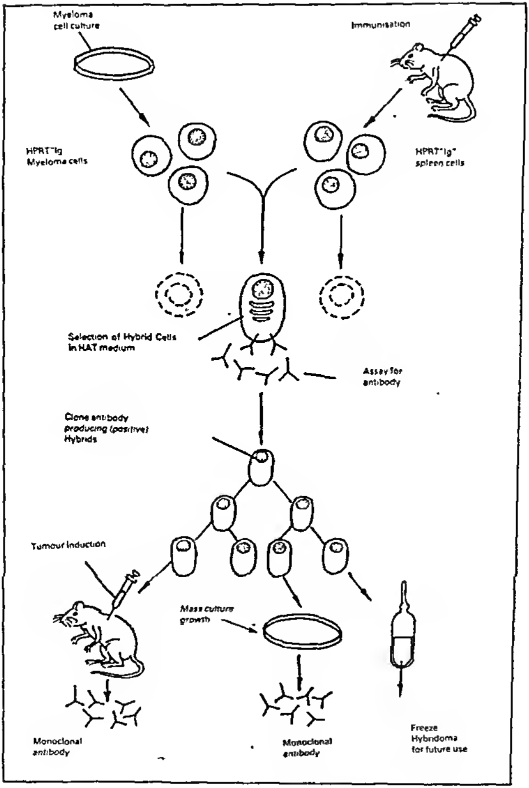

Fig. 16 3 Monoclonal antibody production by hybridoma

protein and other T cell dependent antigens develop early, while responses to polysaccharide and other T cell independent antigens develop only later, usually by two years. Most IgG1 antibodies to polysaccharides are of IgG2 type, and IgG2 producing B cells are the last to mature during infancy.

Nutritional status. Malnutrition affects the immune response adversely, though serum components necessary for immunity are conserved selectively till nutritional deficiency becomes marked. Protein calorie malnutrition suppresses both humoral and cellular immune responses, the latter more severely. Deficiencies of aminoacids (tryptophan, phenyl alanine, methionine, glycine, isoleucine) and vitamins (vitamin A, and B group factors riboflavine, pyridoxine, pantothenic acid, folic acid) have been shown to cause decrease in antibody synthesis.

Route of administration: The humoral immune response is better following parenteral administration of antigen than through oral or nasal routes. Large particulate antigens, such as bacteria or erythrocytes are more effective when injected intravenously whereas soluble antigens are more effective when injected into tissues. The route of administration may also influence the type of antibody produced For production of IgA antibodies, the oral or nasal route is most suitable. Inhalation of pollen antigens induces IgE synthesis whereas the same antigens given parenterally lead to IgG antibodies. With some antigens the route of administration determines whether tolerance or antibody response results. Injection of protein antigens into the mesenteric vein or intrathymically usually induces tolerance. Sulzberger (1929) and Chase (1959) showed that guinea pigs can be rendered specifically tolerant if certain antigens are fed before a parenteral challenge (Sulzberger-Chase Phenomenon). Application of simple chemicals to the skin usually leads to cellular immune response (delayed hypersensitivity) rather than antibody formation.

With some antigens the site of injection seems relevant. It has been reported that hepatitis B vaccine is less immunogenic following gluteal injection than following injection into the deltoid.

Size and number of doses: Antibody response is, to an extent, dose dependent. An antigen is effective only above a minimum critical dose. Further increase in dose enhances the intensity of the antibody response. But beyond a level, increase in the dose of antigen does not improve the antibody response, but may even inhibit it and induce tolerance. Mice injected with 0 5μg of pneumococcal capsular polysaccharide produce specific antibodies, but those injected with 50 μg of the antigen not only fail to form antibody, but may not respond even to subsequent doses of the same antigen. The massive antigenic stimulus appears to swamp the antibody producing system and paralyse it. This phenomenon was designated 'immunological paralysis' by Felton (1949).

The increased antibody respnnse to a secondary antigenic stimulus has already been noticed. With repeated antigen injections the antibody response increases progressively But after a certain stage, no further increase occurs.

The term 'anamnestic reaction' was originally applied to the production in response to an antigenic stimulus, of a heterologous but related antibody that the host had earlier produced. For instance, a person who had been immunised earlier against typhoid bacilli may sometimes produce antityphoid antibodies in response to infection with some other bacterium. This may cause confusion in the serological diagnosis of typhoid fever, but anamnestic reaction can be differentiated from a true secondary response as it is transient. The term anamnestic reaction has been employed by some to refer to the secondary response as well, so that some confusion attaches to this usage.

Multiple antigens: When two or more antigens are administered simultaneously, the effects may vary. Antibodies may be produced against the different antigens just as though they had been

given separately, or antibody response to one or other of the antigens may be enhanced, or the response to one or more of them may be diminished (antigenic competition). When two bacterial vaccines (for example, typhoid and cholera vaccines) are given in a mixed form, the antibody response to each is not influenced by the other. When toxoids are given along with bacterial vaccines (for example, triple vaccine containing diphtheria and tetanus toxoids along with *Bord. pertussis* vaccine) the response to the toxoid is potentiated. When diphtheria and tetanus toxoids are given together, with one in excess, the response to the other is inhibited. When triple antigen is given to a person who had earlier received a primary dose of diphtheria toxoid, the response to tetanus and pertussis antigens will be diminished. Such antigenic competition is important from a practical point of view in immunisation with polyvalent antigens. For optimal effect, the nature and relative porportions of the different antigens in a mixture should be carefully adjusted.

Adjuvants: The term adjuvant refers to any substance that enhances the immunogenicity of an antigen. Adjuvant may confer immunogenicity on nonantigenic substances, increase the concentration and persistence of the circulating antibody, induce or enhance the degree of cellular immunity and lead to the production of 'adjuvant diseases' such as allergic disseminated encephalomyelitis. A variety of substances exhibit adjuvant activity. Some, such as aluminium hydroxide or phosphate and incorporation of protein antigens in the water phase of a water-in-oil emulsion (Freund's incomplete adjuvant), delay the release of antigen from the site of injection and prolong the antigenic stimulus. Others such as silica particles, beryllium sulphate and endotoxins are toxic to macrophages and induce the liberation of lysosomal enzymes, though how this potentiates the immune response is not known. The most potent adjuvant is Freund's complete adjuvant, which is the incomplete adjuvant along with a suspension of killed tubercle bacilli. Besides increasing humoral immune response, it induces a high degree of cellular immunity (delayed hypersensitivity) as well. As it produces a local granuloma it is unsuitable for human use. The adjuvants commonly used with human vaccines are aluminium hydroxide or phosphate, endotoxin and mineral oils.

Immunosuppressive agents: These inhibit the immune response. They are useful in certain situations like transplantation, when it becomes necessary to prevent graft rejection. Examples of immunosuppressive agents are X-irradiation, radiomimetic drugs, corticosteroids, antimetabolites and other cytotoxic chemicals, and antilymphocytic serum.

Sublethal whole body irradiation suppresses antibody response. When antigenic stimulus follows 24 hours after irradiation, antibody production does not occur, whereas if the antigen is administered 2-3 days before irradiation, the antibody response is actually enhanced. The primary response is more radiosensitive than the secondary response.

Radiomimetic drugs are agents with an action resembling that of X-rays. They belong in general to the class of alkylating agents (e.g., cyclophosphamide, nitrogen mustard). In man, cyclophosphamide given for three days after the antigen, completely suppresses the antibody response. It is much less effective when given before the antigen.

Corticosteroids cause depletion of lymphocytes from the blood and lymphoid organs. They also stabilise the membranes of cells and lysosomes, inhibiting histamine release and the inflammatory response. They suppress antibody formation in the rat, mouse and rabbit, but are much less effective in the guinea pig, monkey and man. Therapeutic doses have little effect on the antibody formation in man. They inhibit the induction and manifestations of delayed hypersensitivity in man.

Antimetabolites are substances that interfere with the synthesis of DNA, RNA or both and thus inhibit cell division and differentiation

necessary for humoral and cellular immune responses. They include folic acid antagonists (methotrexate) alkylating agents (cyclophosphamide) and analogues of purine (6-mercaptopurine, azathioprine), cytosine (cytosine arabinoside) and uracil (5-Fluorouracil). Many antimetabolites find clinical application in the prevention of graft rejection.

The drug most widely used now for immunosuppression is cyclosporine. It is a cyclic polypeptide which is not cytotoxic for lymphocytes and has no antimitotic activity. It selectively inhibits helper T cell activity.

Antilymphocyte serum (ALS) is a heterologous antiserum raised against lymphocytes or thymocytes. ALS for human use is raised in horses. Antibodies to other antigens such as erythrocytes are removed by selective absorption. While all other immunosuppressive agents have undesirable side effects, ALS is devoid of any action other than on lymphocytes. The antibody class active in ALS is IgG, the IgM antibody being inactive. ALS acts primarily against T lymphocytes and therefore specifically on cell mediated immunity. Humoral antibody response to thymus dependent antigens may be inhibited, but the response to thymus independent antigens is unaffected and may even be enhanced. ALS acts only against lymphocytes in circulation and not cells in lymphoid organs. As ALS is a foreign protein, its effect is decreased on repeated administration, which may lead to serum sickness and other hypersensitivity reactions.

Effect of antibody: The humoral immune response to an antigen can be suppressed specifically by passive administration of the homologous antibody. The action appears to be by a feedback mechanism. The primary response is more susceptible to inhibition than the secondary response. The antibody may also combine with the antigen and prevent its availability for the immunocompetent cells. The inhibitory effect of a passively administered antibody on the humoral immune response has been applied in the prevention of Rh sensitisation in Rh-negative women carrying Rh-positive fetuses. This is achieved by administration of anti-Rh globulin immediately following delivery (within 72 hours).

This effect is also relevant in the practice of combined immunisation as in diphtheria and tetanus. In such cases, the toxoid and antitoxin should be given at separate sites. Adsorbed toxoid should be used as the inhibitory effect is much less than with fluid toxoid.

CELLULAR IMMUNE RESPONSE

The term 'cell mediated immunity' (CMI) refers to the specific immune responses that do not involve antibodies. Till recently, the only facet of CMI that was investigated to any extent was the phenomenon of delayed hypersensitivity (DH). This apparent neglect was not because CMI is less important than humoral antibody response, but for the reason that the only method available for detection of CMI was the demonstration of DH by the skin test. In recent years, there has been very great progress in understanding the mechanism, scope and significance of CMI. It is now realised that CMI is probably more vital to health and even for life than humoral immunity. The first description of a CMI response was the observation by Jenner (1798) that inoculation of vaccinia virus in an immune individual led to a local erythematous papule in 24–72 hours. He called this the 'reaction of immunity'. Koch (1890) described the exaggerated cutaneous reaction of tuberculous guinea pigs to intradermal injection of the tubercle bacillus or a protein extract of the bacillus (tuberculin). Thereafter the tuberculin test became the paradigm for DH. The term 'delayed hypersensitivity' refers to the appearance of the skin lesion 48–72 hours after administration of the antigen. The lesion is an indurated nodule with infiltration by mononuclear cells. DH was found to be immunologically specific, but it did not have any relation to antibodies and could not be transferred passively

by serum. The cellular basis of DH was shown by Landsteiner and Chase (1942) by its passive transfer in guinea pigs by the injection of leucocytes from sensitised donors. With the recognition of the two-component concept of immunity. DH and other types of CMI were found to be mediated by T lymphocytes. A variety of techniques are now available for detection of CMI, though they lack the sensitivity and precision of antibody assays for humoral immunity.

Scope of CMI

CMI is known to subserve the following immunological functions:

1. Delayed hypersensitivity.
2. Immunity in infectious diseases caused by obligate and facultative intracellular parasites These include infections with bacteria (e.g., tuberculosis, leprosy, listeriosis, brucellosis), fungi (e.g., histoplasmosis, coccidioidomycosis, blastomycosis), protozoa (e.g., leishmaniasis, trypanosomiasis) and viruses (e.g., measles, mumps).
3. Transplantation immunity and graft-versus-host reaction.
4. Immunological surveillance and immunity against cancer.
5. Pathogenesis of certain autoimmune diseases (e.g., thyroiditis, encephalomyelitis).

Induction of CMI

The nature of the antigenic stimulus is important in the induction of CMI. It is developed best following infections with intracellular parasites. Killed vaccines and other nonliving antigens do not induce CMI unless administered with Freund type adjuvants. Application of certian chemicals to the skin induces DH. The reason for the special requirements for the induction of CMI is not known. An understanding of the special requirements for CMI induction may lead to the development of killed vaccines for diseases like tuberculosis and leprosy in which CMI is vital.

Induction of CMI consists of specifically sensitising T lymphocytes against the antigen. When a sensitised cell comes into contact with the antigenic determinant, it undergoes blast transformation and clonal proliferation. The activated lymphocytes release biologically active products (lymphokines) which are responsible for the manifestations of CMI. Macrophages and other mononuclear cells, under the effect of lymphokines, effect the destruction of microorganisms and other processes involved in CMI.

Lymphokines

Biologically active substances released by activated T lymphocytes are known as lymphokines. They are regulatory proteins whose main function appears to be regulation of the immune response and the growth and functions of cells of the reticuloendothelial system Some lymphokines affect the growth of tumour cells also. Similar proteins are produced by monocytes/macrophages (monokines) and other cells. Together they represent a family of hormone-like substances concerned with regulatory interaction among cells (cytokines).

Lymphokines have been named based on the biological effects they produce. Various lymphokines and their biological activities are listed below.

Affecting macrophages: 1. Migration Inhibiting Factor (MIF): Inhibits the migration of normal macrophages. Migration of peripheral leucocytes and of lymph node cells can also be inhibited by lymphokines, but the nature of this factor is not clear.

2. Macrophage Activating Factor: Increases macrophages' adherence to glass, phagocytic capacity, certain biochemical activities, bacteriostatic and tumoricidal activity. It is indistinguishable from MIF.

3. Macrophage Aggregation Factor (MAF): Causes aggregation of macrophages in suspension.

4. Macrophage Chemotactic Factor (MCF): Causes migration of macrophages through millipore membranes along a gradient.

5. Macrophage Resistance Factor (MRF): Renders macrophages nonspecifically resistant to infection with certain pathogens.

6. Macrophage Antispreading Factor: Inhibits spreading of macrophages on glass surfaces.

7. Cytophilic Factor (CF): Confers on macrophages specific reactivity with antigen.

Affecting lymphocytes: 1. Blastogenic or Mitogenic Factor (BF or MF): Induces blast cell transformation and incorporation of tritiated thymidine in lymphocytes.

2. Potentiating Factor (PF): Augments transformation in mixed lymphocyte culture or antigen stimulated culture.

3. Cell Cooperation or Helper Factor (CC or HF): Increases number or rate of antibody forming cells.

4. T cell growth factor, this is required for continuous growth of T cells *in vitro*.

5. B cell Growth Factor (BGF): This stimulates the growth of B cells.

6. T cell Replacement Factor (TRF): This stimulates immunoglobulin production by B cells.

Affecting granulocytes. 1. Inhibition Factor (IF): Inhibits migration of leucocytes.

2. Chemotactic Factor (CF). Causes migration of granulocytes along a gradient.

3. Colony Stimulating Factor (CSF): Stimulates growth of granulocytes and macrophages.

Affecting cultured cells: 1. Lymphotoxin: Cytotoxic for certain cultured cells.

2. Proliferation Inhibiting Factor or Colony Inhibiting Factor (PIF or CLIF): Inhibits proliferation of cultured cells without lysing them

3. Gamma interferon: Protects cells against virus infection, has an inhibitory effect on malignant cells and a regulatory effect on the immune response.

4. Tumour Necrosis Factor (TNF): Producing lysis of tumour cells.

Producing in vitro effects: 1. Skin Reactive Factor (SRF): Induces the skin lesion of DH; possibly a combination of several factors.

2. Inflammatory Factor (IF): Causes increased vascular permeability and other inflammatory changes on normal skin.

3. Transfer Factor (TF): Mediates passive transfer of CMI.

4. Tissue Factor (Procoagulant Factor): This, when incubated with factor VIII deficient plasma is able to correct prolonged clotting time.

5. Osteoclast Activating Factor

Interleukins

Many lymphokines have not been adequately characterised. Their names indicate their demonstrated biological effects, but as most lymphocytes exhibit multiple biological activities, the descriptive names can be misleading. Therefore a nomenclature has been introduced employing the term *interleukin* followed by a number. The term interleukin signifies substances produced by leucocytes *(leukin)* that regulate other *(inter)* cells. At the 6th International Conference of Immunology in 1986, it was agreed that a new lymphokine or cytokine would first be named according to its biological activity, but once it is fully characterised and its aminoacid sequence established, it would be assigned an interleukin number. The following interleukins have been described.

1. Interleukin-1: Originally described as a leucocyte activating factor (LAF) in 1972 and as B cell activating factor (BAF) in 1974, this cytokine was renamed interleukin-1 in 1979. IL-1 is a stable polypeptide retaining its activity at temperatures upto 56°C and pH range 3–11. Two structurally related IL-1 species have been described, termed alpha and beta IL-1. Initially observed to be secreted by monocytes and macrophages, IL-1 is now known to be produced by virtually all nucleated cells. Production of IL-1 is induced by antigens, toxins, injury and inflammatory processes.

IL-1 activates T-cells and promotes synthesis of lymphokines. It stimulates B cell proliferation, differentiation and antibody synthesis. It acts as a co-factor for haematopoietic growth factors, and induces fever, ACTH release, neutrophilic and other systemic acute phase responses. It mediates inflammatory processes and augments nonspecific resistance to infection.

2. *Interleukin-2*: In 1976, a polypeptide factor produced by activated T cells was described, which induced T cells to proliferate and enabled them to be maintained in continuous culture. Known originally as T cell growth or activation factor (TCGF), it has since been named IL-2. It is a 15,000 M.W. glycoprotein which is a powerful modulator of immune response. Mere presence of IL-2 is not sufficient to activate T cells. It has to be presented with a properly processed antigen from a cell or by a macrophage. The T cell then sprouts surface receptors for IL-2 enabling combination with it and subsequent activation. IL-2 causes induction of T-helper, T-cytotoxic and NK cells. It converts some Null cells into *lymphokine-activated Killer (LAK) cells* which can destroy NK resistant tumour cells. IL-2 has been cloned in *E. coli.* Cloned IL-2 has been used experimentally in cancer patients. Peripheral lymphocytes collected from patients were treated with IL-2, to induce LAK cells, and reinfused, followed by IL-2 infusion. Encouraging results have been claimed in some types of cancers. IL-2 produces adverse reactions due to increased capillary permeability, leading to pulmonary and systemic oedema.

3. *Interleukin-3* is a growth factor for bone marrow stem cells. It stimulates multilineage haematopoiesis.

4. *Interleukin-4* formerly known as B cell stimulating factor-1 (BSF-1) activates resting B cells and acts as a B cell differentiation factor. It also acts as a growth factor for T cells and mast cells. It enhances activity of cytotoxic T cells. It may have a role in atopic hypersensitivity as it augments IgE synthesis.

5. *Interleukin-5* formely known as B cell growth factor II, causes proliferation of activated B cells. It also induces maturation of eosinophils.

6. *Interleukin-6* formerly called B cell stimulating factor-2, is produced by stimulated T cells, fibro-

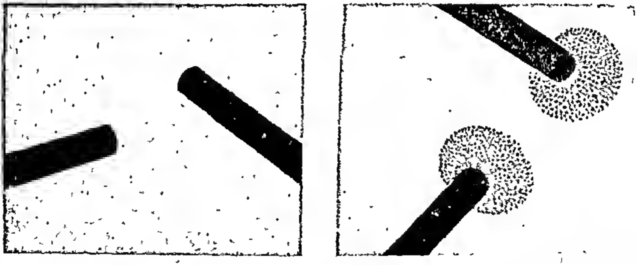

Fig 16 4 Inhibition of migration of macrophage cells. *Left*: Exposure to antigen of cells from sensitised guinea pigs. Migration inhibited. *Right*. Control. Exposure to antigen of cells from normal guinea pigs. Shows foam like migration of macrophages

blasts and macrophages. It induces immunoglobulin synthesis by activated cells and IL-2 receptors on T cells. It also exerts a stimulatory effect on hepatocytes, nerve cells and haematopoietic cells. It has been claimed to have a role in production of some autoimmune processes, particularly rheumatoid arthritis.

Detection of CMI

The only method for detecting CMI till recently was the skin test for DH. A number of *in vitro* correlates of CMI have now become available. These include the lymphocyte transformation test (transformation of cultured sensitised T lymphocytes on contact with the antigen), target cell destruction (killing of cultured cells by T lymphocytes sensitised against them) and the migration inhibiting factor test. The last is most commonly employed. As originally described, this consisted of incubating in a culture chamber, packed peritoneal macrophages in a capillary tube. The macrophages migrate to form a lacy, fan-like pattern. If the macrophages are from a guinea pig sensitised to tuberculoprotein, addition of tuberculin to the culture chamber will inhibit the migration (Fig. 16.4). This has been adapted for clinical use by incubating human peripheral leucocytes in capillary tubes in culture chambers When an antigen to which the individual has CMI is introduced into the culture medium, the leucocytes are prevented from migrating. By comparison with the control, it is possible to make a semiquantitative assessment of the migration inhibition.

Transfer factor

Passive transfer of CMI was first achieved by the injection of viable leucocytes from sensitised donors. Lawrence (1954) reported transfer of CMI in man by the injection of extracts from leucocytes. This extract is known as the 'transfer factor' (TF). The transferred immunity is specific in that CMI can be transferred only to those antigens to which the donor is sensitive.

TF is a dialysable, low molecular weight substance (MW 2000 to 4000), resistant to trypsin, DNA-ase, RNA-ase and freeze thawing. It is stable for several years at –20°C, and in the lyophilised form at 4°C. It is inactivated at 56°C in 30 minutes. It is not antigenic. Chemically, it appears to be a polypeptide-polynucleotide.

TF is highly potent, an extract from 0.1 ml of packed leucocytes being sufficient for transfer. The transferred CMI is systemic and not local at the injected site alone. Following TF injection DH and various *in vitro* correlates of CMI can be demonstrated in the recipient. Humoral immunity is not transmitted by TF. TF transfers CMI to all the antigens to which the donor is sensitive, *en bloc*. It is possible to transfer CMI from the recipient to another in series

The mechanism of action of the TF is not known. TF could be an informational molecule or a specific gene derepressor capable of inducing antigenically uncommitted lymphocytes to produce antigen-specific receptors. TF activity was till recently demonstrable in man only, but it has now been reported in monkeys, guinea pigs and mice.

TF has several applications. It has been used to restore immune capacity in patients with T cell deficiency (Wiskott-Aldrich Syndrome). It has also been used in the treatment of disseminated infections associated with deficient CMI (lepromatous leprosy, tuberculosis, mucocutaneous candidiasis). It has been employed in the treatment of malignant melanoma and may be beneficial in other types of cancer as well. It use has been suggested in some autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis) and diseases of unknown aetiology (sarcoidosis, multiple sclerosis).

Interactions between humoral and cellular immune processes

Humoral and cellular immune processes generally act in conjunction, but may sometimes be antagonistic. Some interactions between the two are dealt with below:

Immunological enhancement: This is a process in which humoral antibodies protect transplants and tumour cells from the effect of CMI.

Immune deviation: This refers to the process whereby prior immunisation with an antigen prevents development of CMI when subsequently challenged with the same antigen in a form that ordinarily would induce CMI.

Jones-Mote hypersensitivity: This is a transient development of DH on immunisation with protein antigens in the absence of adjuvants. This is followed by the development of antibody response. This differs from classical DH in the rapid appearance of hypersensitivity (12–24 hours) and its transitory nature. It differs histologically also in that basophils are present in the infiltrate. The term 'cutaneous basophil hypersensitivity' has therefore been suggested for this reaction.

T cell modulation of B cell function: Though B cells are responsible for the humoral immune response, T cells perform helper or amplifier functions in this connection. The switch over from IgM to IgG production requires the participation of activated T cells. They also exert regulatory or suppressor effect on B cell functions. Overactivity of suppressor cells may lead to immunodeficiencies while inadequate suppressor cell activity may result in autoimmunity.

IMMUNOLOGICAL TOLERANCE

Immunological tolerance or immunological unresponsiveness is the condition in which contact with an antigen specifically abolishes the capacity to mount an immune response against that particular antigen when it is administered subsequently. This nonreactivity is specific to the particular antigen, immune reactivity to other antigens being unaffected.

The first example of immunological tolerance was the observation by Owen (1945) of erythrocyte chimerism in dizygotic cattle twins, each of the twins having erythrocytes of its own and the other's blood groups. As dizygotic twins are genetically dissimilar, they do not ordinarily accept transplants from each other, but such transplants survive in cattle twins. The reason for this tolerance was shown to be the sharing of the same placental blood supply by the twins during intrauterine life. Based on this observation, Burnet and Fenner (1949) suggested that the unresponsiveness of individuals to self-antigens was due to the contact of the immature immunological system with self-antigens during embryonic life. Any antigen that comes into contact with the immunological system during its embryonic life would be recognised as a self-antigen and would not induce any immune response. They postulated that tolerance could be induced against foreign antigens if they were administered during embryonic life. This was proved experimentally by Medawar and his colleagues (1953) using two strains of syngeneic mice. When skin graft from one inbred strain of mice (CBA) is applied on a mouse of another strain (A), it is rejected. But if CBA cells are injected into fetal or newborn A strain mice, the latter when they grow up will freely accept skin grafts from CBA mice. The content of self-antigen appears to have been enlarged by contact with a foreign antigen during embryonic life. This phenomenon is called 'specific immunological tolerance'.

Development of tolerance is not confined to the embryo or newborn, but can occur in adults also. Tolerance may be total or partial, short-lived or long lasting. The induction, degree and duration of tolerance depend on the species and immunocompetence of the host, nature and dose of the antigen and the route of administration. (1) Rabbits and mice can be rendered tolerant more rapidly than guinea pigs and chickens. Strain differences in tolerance induction are seen within species. (2) The higher the degree of immunocompetence of the host, the more difficult it is to induce tolerance. It is for this reason that embryos and newborns are particularly susceptible for induction of tolerance. Tolerance can be induced in adults in whom immunocompetence is tempora-

rily interrupted by immunosuppressive agents. Induction of tolerance is very difficult in adults already immunised against the antigen.

The physical state of the antigen is important. Soluble antigens and haptens are more tolerogenic than particulate antigens. The tolerogenicity of an antigen can be modified by certain procedures. When human gamma globulin is heat aggregated, it is highly immunogenic in mice, but when de-aggregated, it is tolerogenic. Solutions of serum proteins centrifuged at high speed separate into tolerogenic supernatant and immunogenic sediment fractions. The induction of tolerance is dose-dependent. There is a threshold dose below which tolerance is not induced. Further increase in dose increases the duration of tolerance. With certain antigens, tolerance can be induced by two types of doses, one high and the other low, with intermediate doses producing immunity instead of tolerance. These are known as 'high zone' and 'low zone' tolerance. A special type of high zone tolerance is Felton's immunological paralysis. The duration of tolerance is variable. Tolerance can be prolonged by repeated tolerogenic stimuli. The route of administration that induces tolerance best is that whereby antigen equilibrates throughout the extra- and intravascular compartments. With antigens that do not equilibrate readily or are rapidly eliminated, the route of choice is intravenous. Certain haptens that are immunogenic in guinea pigs by the intradermal route are tolerogenic orally or intravenously.

Tolerance can be overcome spontaneously or by an injection of cross reacting immunogens. For example, tolerance to bovine serum albumin in rabbits can be abolished by immunisation with cross reacting human serum albumin. In general, tolerance to living agents is more lasting than that to nonliving substances. Naturally occurring tolerance is found in certain virus infections as in congenital rubella and cytomegalovirus infections in which there is persistent viraemia with a decreased ability for the production of neutralising antibodies (persistent tolerant infection). In lymphocytic choriomeningitis infection in carrier mice, the virus may persist in virtually all the cells and tissues and be transmitted vertically to the offspring without any demonstrable immune response or pathogenic effect. When the tolerance is interrupted by an induction of antibody or by an injection of sensitised lymphocytes, disease results.

The mechanism of tolerance is not clear. In specific immunological tolerance in embryonic life, the clones of cells responding to the particular antigen are believed to be annihilated by contact with the antigen. This is the 'central mechanism' of tolerance induction. In other instances the mechanism may be 'afferent block' in which access of the antigen to immunocompetent cells is interfered with, or 'efferent block' in which the antibody synthesised is neutralised or destroyed. T and B lymphocytes appear to possess different sensitivity to tolerance induction, the former being more susceptible. It is probably for this reason that low zone tolerance is seen with thymus-dependent antigens like serum proteins. With thymus independent antigens, tolerance depends on rendering B cells unresponsive and this requires a higher dose.

Tolerance to humoral and cellular types of immunity is usually induced simultaneously. 'Split tolerance', where unresponsiveness is established for one parameter of the immune response and not to the other, can be induced by special techniques. In guinea pigs, DH to tuberculin can be inhibited, without affecting the production of a circulating antibody, by injection of tuberculoprotein prior to immunisation with BCG.

THEORIES OF IMMUNE RESPONSE

A succession of theories have been put forward, from time to time, in order to explain the versatility, specificity, memory and other features of the immune response. Theories of immunity fall into two categories: *instructive* and *selective*. The instructive theories postulate that an immunocompetent cell is capable of synthesising antibodies of any specificity. The antigen encounters

an immunocompetent cell and *instructs* it to produce the complementary antibody. Instructive theories were proposed by chemists who were more concerned with explaining the physicochemical aspects of specificity than with biological principles of immune processes. Selective theories, on the contrary, shift the emphasis from the antigen to the immunocompetent cell. They postulate that immunocompetent cells have only a restricted immunological range. The antigen exerts only a *selective* influence by stimulating the appropriate immunocompetent cell to synthesise an antibody.

1) *Side chain theory*: The first convincing theory of immune response was the 'side chain' theory proposed by Ehrlich (1900). Cells were considered to have surface 'receptors' capable of reacting with substances having complementary 'side chains'. The physiological significance of such receptors was in anchoring nutrients to cells prior to their assimilation. When foreign antigens are introduced into the body, they combine with those cell receptors, which have a complementary 'fit'. This inactivates the receptors and interferes with absorption of nutrients. As a compensatory mechanism, there is an overproduction of the same type of receptors, which spill over into the blood and circulate as antibodies. This was the first of the selection theories. It explained elegantly the specificity of the antibody response. But when Landsteiner demonstrated that antibodies could be formed not only against natural antigens, but also against various synthetic chemicals, this theory was abandoned. It was believed that an impossibly large number of receptors would be needed to account for the seemingly endless scope of antibody specificity. It is, however, remarkable how closely Ehrlich anticipated modern views on the immune response.

2) *Direct template theories*: Instructive theories were proposed by Breinl and Haurowitz (1930), Alexander (1931) and Mudd (1932). According to these the antigen (or the antigenic determinant) enters antibody forming cells and serves as a 'template' against which antibody molecules are synthesised so that they have combining sites complementary to the antigenic determinant. These are therefore known as 'direct template' theories. Pauling (1940) presented a more detailed model suggesting that specificity was determined by the folding of the antibody polypeptide chains to form a tertiary structure fitting the antigenic determinant.

3) *Indirect template theory*: Burnet and Fenner (1949) proposed this instructive theory to explain the synthesis of antibody as an adaptive protein. They postulated that the entry of the antigenic determinant into the antibody producing cell produced in it a heritable change so that a 'genocopy' of the antigenic determinant was incorporated in its genome and transmitted to the progeny cells (indirect template). This theory explained specificity and the secondary response, but became untenable with advances in the molecular biology of protein synthesis. Burnet and Fenner were the first to explain the nonantigenicity of self-antigens by postulating the embryonic recognition of 'self-markers'.

4) *Natural selection theory*: Jerne (1955) reintroduced the concept of the selective function of antigens in his natural selection theory. This postulated that about a million globulin (antibody) molecules were formed in embryonic life, which covered the full range of antigenic specificities. These globulins were the 'natural antibodies'. When an antigen was introduced, it combined selectively with the globulin that had the nearest complementary 'fit'. The globulin, with the combined antigen, homed to antibody forming cells and stimulated them to synthesise the same kind of antibody. Here, selection was postulated at the level of the antibody molecule. It did not explain the fact that immunological memory resides in the cells, and not in serum.

5) *Clonal selection theory*: Burnet (1957) proposed this theory which shifted immunological specific-

ity to the cellular level. A similar theory was also proposed by Talmage.

According to the clonal selection hypothesis, during immunological development, cells capable of reacting with different antigens were formed by a process of somatic mutation. Clones of cells that had immunological reactivity with self-antigens were eliminated during embryonic life. Such clones are called 'forbidden clones'. Their persistence or development in later life by somatic mutation could lead to autoimmune processes. Each immunocompetent cell was capable of reacting with one antigen (or a small number of antigens) which could recognise and combine with antigens introduced into the body. The result of contact with the specific antigen was cellular proliferation to form clones synthesising the antibody.

The clonal selection theory is more widely accepted than other theories, though it is unable to account for all the features of the immune response. A variety of modifications and alternate theories have been proposed in recent times, but none has succeeded in explaining all that is known of immunity

As an explanation for the mechanism of regulation of antibody response, Jerne has postulated the *network hypothesis*. The variable region of an immunoglobulin molecule carrying the antigen combining site is different in different antibodies. The distinct aminoacid sequences at the antigen combining site and adjacent parts of the variable region are termed *'idiotypes'*. The idiotype can, in turn, act as an antigenic determinant and induce antidiotypic antibodies. These in turn can induce antibodies to them and so on, forming an idiotype network which is postulated to regulate the amount of antibodies produced and the number of antibody forming cells in action.

The genetic basis of antibody diversity has been clarified recently. An individual has the capacity to produce millions of species of antibody molecules. To have each such antibody molecule to be coded for by a separate gene would require millions of genes to be set apart for antibody production alone. This would be obviously impossible. The phenomenon of split genes explains this. The genetic information for the synthesis of an immunoglobulin molecule is not present in a continuous array of codons. Instead, this information occurs in several discontinuous stretches of DNA, each coding for separate regions of antibody molecule. As the constant regions are identical for immunoglobulins of any one type, there need be only one gene or a few genes for each constant region, as against a very large number of genes for the variable regions. By shuffling the genes during the development of lymphocytes, antibody specificity can be amplified to cover billions of antigenic determinants.

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17 Immunodeficiency Diseases

Immunodeficiency diseases are conditions where the defence mechanisms of the body are impaired, leading to repeated microbial infections of varying severity and sometimes enhanced susceptibility to malignancies. Deficiencies of defence mechanisms may involve specific immune functions — humoral immunity, cell mediated immunity or both — or nonspecific mechanisms such as phagocytosis and complement, which augment and act in conjunction with specific immune processes. Immunodeficiencies may be classified as primary or secondary. Primary immunodeficiencies result from abnormalities in the development of the immune mechanisms. Secondary immunodeficiencies are consequences of disease, drugs, nutritional inadequacies and other processes that interfere with the proper functioning of the mature immune system.

PRIMARY IMMUNODEFICIENCIES

The established types of primary immunodeficiency syndromes are listed in Table 17.1. Though primary deficiencies of specific immunity can conveniently be classified as those affecting B cell responses, T cell responses, or both, it must be realised that there is considerable overlapping due to the intimate interaction between the B cell and the T cell systems. For instance, T cell deficiencies involving helper or suppressor T cells will have a profound effect on antibody response.

Humoral immunodeficiencies

X-linked agammaglobulinaemia: This syndrome described by Bruton (1952) is the first immunodeficiency disease to have been recognised. It is seen only in male infants. Manifestations are not apparent till about six months of age due to the passive protection afforded by maternal antibodies. The disease presents as recurrent serious infections with pyogenic bacteria, particularly with pneumococci, streptococci, meningococci *Pseudomonas* and *H. influenzae*. Patients respond normally to viral infections such as measles and chickenpox, though there have been reports of paralytic poliomyelitis and progressive encephalitis following immunisation with live virus vaccines or exposure to wild virus. As a general rule, live microbial vaccines should not be given to children with any type of primary immunodeficiency.

All classes of immunoglobulins are grossly depleted in the serum, the IgG level being less than a tenth, and IgA and IgM less than a hundredth of the normal level. Tonsils and adenoids are atrophic. Lymph node biopsy reveals a depletion of cells of the bursa-dependent areas. Plasma cells and germinal centres are absent even after antigenic stimulation. There is a marked decrease in the proportion of B cells in circulation. Antibody formation does not occur even after injections of antigens.

Cell mediated immunity is not affected. Delayed hypersensitivity of tuberculin and contact dermatitis types can be demonstrated. Allograft rejection is normal. Arthritis, haemolytic anaemia and atopic manifestations are frequently observed. However, the wheal and flare response of atopic hypersensitivity cannot be demonstrated.

The incidence of this condition has been reported to be one in a hundred thousand population in the United Kingdom. Its management consists in the maintenance of an adequate level of immunoglobulins. This can be achieved by the initial administration of 300 mg of gamma globulin per kg of body weight in three doses followed by monthly injections of 100 mg per kg. The slow fractional catabolic rate of IgG in this condition enables the maintenance of effective levels with this dosage. Commercial preparations of gamma globulin contain only traces of IgA and IgM. To provide these, whole plasma infusions have been employed, the donors being tested for hepatitis and other transmissible infections.

Transient hypogammaglobulinaemia of infancy

This is due to an abnormal delay in the initiation of IgG synthesis in some infants. Maternal IgG is slowly catabolised in the newborn and reaches a level of 200 mg per 100 ml by the second month. Ordinarily, the infant begins synthesising its own IgG by this age. When there is a delay, immunodeficiency occurs. Recurrent otitis media and respiratory infections are the common diseases found in this condition. Spontaneous recovery occurs between 18 and 30 months of age. It may be found in infants of both sexes. Treatment with gamma globulin may be required in some cases, but it is not recommended prophylactically, as it may contribute to prolongation of immunodeficiency by a negative feedback inhibition of IgG synthesis.

Common variable immunodeficiency

This common form of immunodeficiency is also known as *late onset hypogammaglobulinaemia* because it usually manifests only by 15–35 years of age. It is characterised by recurrent pyogenic infections and an increased incidence of autoimmune disease. Malabsorption and giardiasis are common. The total immunoglobulin level is usually less than 300 mg per 100 ml, with IgG less circulation in normal numbers, but they appear defective in being unable to differentiate into plasma cells and secrete immunoglobulins. Increased suppressor T cell and diminished helper T cell activity have been proposed as a cause of this disorder. Treatment is by administration of gamma globulin preparations intramuscularly or intradermally.

Selective immunoglobulin deficiencies

In these conditions, there occurs selective deficiency of one or more immunoglobulin classes, while the others remain normal or elevated. These 'dysgammaglobulinaemias' are common and have been reported to be present in about one per cent of all patients with recurrent infections. Isolated IgA deficiency is the most common condition in this group, with a reported incidence of about 0.2 per cent in normal populations. These patients exhibit increased susceptibility to respiratory infection and steatorrhoea. IgA deficiency is often accompanied by atopic disorders. Anti-IgA antibodies are present in many of these patients.

Selective IgM deficiency has been found to be associated with septicaemia. Deficiencies of IgG subclasses have been observed in relation with chronic progressive bronchiectasis.

Immunodeficiencies with hyper-IgM: In this group of immunodeficiencies, some of which are X-linked and some inherited as autosomal recessive, low IgA and IgG levels are seen with elevated IgM. The IgM molecules appear to be normal in structure and possess antibody activity. Patients show enhanced susceptibility to infections and autoimmune processes such as thrombocytopaenia, neutropaenia, haemolytic anaemia and renal lesions. Some patients develop malignant infiltration with IgM-producing cells. Elevated IgM level with immunodeficiency is sometimes seen in congenital rubella.

Transcobalamin II deficiency: In this disorder, inherited as autosomal recessive, patients show

TABLE 17 1

Classification of primary immunodeficiency syndromes

A. Disorders of *Specific Immunity*.

- I. Humoral Immunodeficiencies (B cell defects).
  - a. X-linked agammaglobulinaemia
  - b. Transient hypogammaglobulinaemia of infancy.
  - c. Common variable immunodeficiency (late onset hypogammaglobulinaemia).
  - d. Selective immunoglobulin deficiencies (IgA, IgM or IgG subclasses)
  - e. Immunodeficiencies with hyper-IgM.
  - f. Transcobalamin II deficiency.
- II. Cellular Immunodeficiencies (T cell defects).
  - a. Thymic hypoplasia (DiGeorge's syndrome)
  - b. Chronic mucocutaneous candidiasis
  - c. Purine nucleoside phosphorylase (PNP) deficiency.
- III. Combined Immunodeficiencies (B and T cell defects)
  - a. Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof syndrome)
  - b. Ataxia telangiectasia.
  - c. Wiskott-Aldrich Syndrome.
  - d. Immunodeficiency with thymoma
  - e. *Immunodeficiency with short-limbed dwarfism*
  - f. Episodic lymphopaenia with lymphocytotoxin
  - g. Severe combined immunodeficiencies.
    - 1. 'Swiss type' agammaglobulinaemia.
    - 2. Reticular dysgenesis of de Vaal
    - 3. Adenosine deaminase (ADA) deficiency

B. Disorders of Complement.

- a. Complement component deficiencies
- b. Complement inhibitor deficiencies

C. Disorders of Phagocytosis

- a. Chronic granulomatous disease
- b. Myeloperoxidase deficiency.
- c. *Chediak-Higashi Syndrome*
- d. Leucocyte G6PD deficiency.
- e. Job's syndrome
- f. Tuftsin deficiency.
- g. Lazy leucocyte syndrome.
- h. Hyper-IgE syndrome
- i. *Actin-binding protein deficiency*
- j. Shwachman's disease.

Severe CAW.

CAW

JIS

CMC, JLT, LHAS.

CMC JLT SHA

metabolic effects of vitamin B_{12} deficiency including megaloblastic anaemia and intestinal villous atrophy. Associated immunological defects are depleted plasma cells, diminished immunoglobulin levels and impaired phagocytosis. Treatment with vitamin B_{12} has been reported to restore haematopoietic, gastrointestinal and B cell functions, but not phagocytic activity.

Cellular Immunodeficiencies

Thymic hypoplasia (DiGeorge's syndrome): This is a developmental defect involving the endodermal derivatives of the third and fourth pharyngeal pouches, which leads to aplasia or hypoplasia of the thymus and parathyroid glands. It does not appear to be hereditary and does not show a familial incidence. It is probably due to some intrauterine infection or other complication. It is usually associated with Fallot's tetrology and other anomalies of the heart and the great vessels, and a characteristic facial appearance. Neonatal tetany is present. Patients who survive the neonatal period show enhanced susceptibility to viral, fungal and bacterial infections, which ultimately prove fatal.

The immunodeficiency primarily involves cell mediated immunity. The thymus dependent areas of lymph nodes and spleen are depleted of lymphocytes. Circulating T cells are reduced in numbers. Delayed hypersensitivity and graft rejection are depressed. The humoral immune mechanism is largely unaffected. Antibody response to primary antigenic stimuli is normal, but secondary response to many antigens is impaired. Transplantation of fetal thymus tissue has been reported to restore the immunological function.

Chronic mucocutaneous candidiasis: This constitutes an abnormal immunological response to *Candida albicans*. Patients develop severe chronic candidiasis of the mucosa, skin and nails. They do not show increased susceptibility to other infections, but often have endocrinopathies. Cell mediated immunity to candida is deficient. In some cases there is a total failure of T cell response to any test antigen. Delayed hypersensitivity to candida antigens is absent, but circulating antibodies to them are found in high titre. Intracellular killing of candida is defective. Transfer factor therapy, along with amphotericin B, has been reported to be effective.

Purine nucleoside phosphorylase (PNP) deficiency: The enzyme purine nucleoside phosphorylase is involved in the sequential degradation of purines to hypoxanthine and finally to uric acid. Patients who have PNP deficiency as an autosomal recessive inherited trait show decreased cell mediated immunity and recurrent or chronic infections. They usually present with hypoplastic anaemia and recurrent pneumonia, diarrhoea and candidiasis. A low serum uric acid may point to the diagnosis.

Combined Immunodeficiencies

Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof syndrome): The term Nezelof syndrome has been rather loosely applied to a group of disorders, probably of varied aetiology, where depressed cell mediated immunity is associated with selectively elevated, decreased or normal levels of immunoglobulin. Consistent features are marked deficiency of T cell immunity and varying degrees of deficiency of B cell immunity. Patients are susceptible to recurrent fungal, bacterial, viral and protozoal diseases. Abundant numbers of plasma cells are seen in the spleen, lymph nodes, intestines and elsewhere in the body. Thymic dysplasia occurs with lymphoid depletion. Autoimmune processes such as haemolytic anaemia are common. In spite of normal levels of immunoglobulins, antigenic stimuli do not induce antibody formation.

Histocompatible bone marrow transplantation, transfer factor and thymus transplantation have been used for treatment, with success in some cases. Adequate antimicrobial therapy is essential.

Ataxia telangiectasia: This is a hereditary condition transmitted in autosomal recessive mode, where combined immunodeficiency is associated with cerebellar ataxia, telangiectasia, ovarian dysgenesis and chromosomal abnormalities. The earliest signs are ataxia and chorioathetoid movements which are noticed usually in infancy. Telangiectasia involving conjunctiva, face and

other parts of the body usually appears at five or six years of age. Death occurs due to sinopulmonary infection early in life, or malignancy in the second or third decade. The majority of patients lack serum and secretory IgA and some possess antibody to IgA. IgE deficiency is also frequent. Cell mediated immunity is also defective, resulting in an impairment of delayed hypersensitivity and graft rejection. The disease is progressive, with both neurological defects and immunodeficiency becoming more severe with time. Transfer factor and fetal thymus transplants have been tried with some benefit.

Wiskott-Aldrich syndrome: This is an X-linked disease characterised by eczema, thrombocytopaenic purpura and recurrent infections. Affected boys rarely survive the first decade of their life, death being due to infection, haemorrhage or lymphoreticular malignancy. Cell mediated immunity undergoes progressive deterioration associated with cellular depletion of the thymus and the paracortical areas of lymph nodes. Serum IgM level is low, but IgG and IgA levels are normal or elevated. Isohaemagglutinins are absent in the serum. The humoral defect appears to be specific inability to respond to polysaccharide antigens. Bone marrow transplantation and transfer factor therapy have been found to be beneficial.

Immunodeficiency with thymoma: This syndrome, occurring usually in adults, consists of a benign thymic tumour, impaired cell mediated immunity and agammaglobulinaemia. Aplastic anaemia is a frequent accompaniment. This is of historical importance as one of the experiments of nature which suggested the immunological function of the thymus.

Immunodeficiency with short-limbed dwarfism: The features of this condition are a distinctive form of short-limbed dwarfism, ectodermal dysplasia, thymic defects and enhanced susceptibility to infection. These defects are apparently inherited as autosomal recessives.

Episodic lymphopaenia with lymphocytotoxin: In this syndrome there occurs an episodic, but profound, depression of T cell function by the action of a circulating complement dependent lymphocytotoxin. The toxin appears to be an antilymphocyte antibody. The patients lack 'immunological memory' so that the secondary antibody response is abolished. The disease is familial.

Severe combined immunodeficiencies: These include many syndromes with severe deficiency of both humoral and cell mediated immune responses. They are inherited in the autosomal recessive mode and the primary defects are at the level of the early precursors of immunocompetent cells in the fetal liver and bone marrow. Many distinct patterns of severe combined immunodeficiency have been described.

In 1958, Swiss workers reported agammaglobulinaemia with lymphocytopaenia and severe defect in cell mediated immunity. This has been referred to as the 'Swiss type agammaglobulinaemia'. The basic defect is presumed to be at the level of the lymphoid stem cell.

The most serious form of combined immunodeficiency is the 'reticular dysgenesis of de Vaal'. Here the defect is at the level of the multipotent haemopoietic stem cell, as a result of which there is a total failure of myelopoiesis leading to lymphopaenia, neutropaenia, thrombocytopaenia, anaemia and bone marrow aplasia. The condition is invariably fatal in the first week of life.

Adenosine deaminase (ADA) deficiency is the first immunodeficiency disease associated with an enzyme deficiency. ADA catalyses the conversion of adenosine to inosine, an important step in purine metabolic pathway. How this deficiency causes immunological impairment is not clear. The range of immunodeficiency varies from complete absence to mild abnormalities of B and T cell functions. The condition is associated with chondrocyte abnormalities which can be made out radiologically.

DISORDERS OF COMPLEMENT

Complement component deficiencies: Genetic deficiencies have been detected for almost all complement components in man. The defects are transmitted as autosomal recessive traits. Haemolytic and other functional activities are completely restored by supplying the deficient factor. Complement component deficiencies have been frequently associated with systemic lupus erythematosus. Recurrent pyogenic infections were found associated with C3 deficiency and neisserial infections with deficiency of C6, C7 and C8.

Complement inhibitor deficiencies: Hereditary angioneurotic oedema is due to a genetic deficiency of C1 inhibitor. This relatively common defect is transmitted as an autosomal dominant. Androgens, aminocaproic acid and its analogue tranexamic acid have been found useful in the management of this condition. Plasma infusions, once recommended for treatment, have been given up as they were found to worsen the condition in some cases.

The rare deficiency of C3b inactivator has been associated with chronic recurrent pyogenic lesions.

DISORDERS OF PHAGOCYTOSIS

Phagocytosis may be impaired either by intrinsic or extrinsic defects. Intrinsic disorders may be due to defects within the phagocytic cell, such as enzyme deficiencies. Extrinsic disorders may be due to a deficiency of opsonic antibody, complement or other factors promoting phagocytosis, or to the effects of drugs or antineutrophil autoantibodies. Phagocytic dysfunction leads to increased susceptibility to infection, ranging from mild recurrent skin infections to overwhelming systemic infection.

Chronic granulomatous disease: This familial disease manifests itself as recurrent infection with low grade pathogens, starting early in life. The progress is chronic and the outcome fatal. Chronic suppurative granulomatous lesions develop in the skin and lymph nodes, along with hepatosplenomegaly, progressive infiltration of lungs and granulomatous septic osteomyelitis. Humoral and cellular immune responses are normal.

The bacteria involved in the recurrent infections are catalase positive pyogenic pathogens such as staphylococci and coliforms. Catalase negative pathogens such as streptococci and pneumococci are handled normally. Leucocytes from the patients are unable to kill catalase-positive bacteria following phagocytosis. The bacteria multiply in the cells and, being protected from antibodies and antibiotics by their intracellular position, set up chronic suppurative infection. The diminished bactericidal capacity of the phagocytic cells is associated with a decrease of some metabolic processes such as oxygen consumption, hexose monophosphate pathway activity and production of hydrogen peroxide. The diminished H_2O_2 production appears to be the major reason for the bactericidal defect. The leucocytes do not undergo degranulation following phagocytosis. The delayed granule rupture and defective release of myeloperoxidase also contribute to inefficient bactericidal activity. Leucocytes from the patient fail to reduce nitroblue tetrazolium (NBT) during phagocytosis. The property has been used as a screening method (NBT test) for the diagnosis of chronic granulomatous disease.

The disease shows two types of inheritance — the more common X-linked type seen in boys and the rare autosomal recessive type seen in girls.

Myeloperoxidase deficiency: In this rare disease, leucocytes have reduced myeloperoxidase. Patients are particularly liable to *Candida albicans* infection.

Chediak-Higashi syndrome: This is a genetic disorder characterised by decreased pigmentation of the skin, eyes and hair, photophobia, nystagmus and giant peroxidase positive inclusions in the cytoplasm of leucocytes. The inclusions may

be the result of autophagocytic activity. The leucocytes possess diminished phagocytic activity. Patients suffer from frequent and severe pyogenic infections.

Leucocyte G6PD deficiency: In this rare disease, leucocytes are deficient in glucose 6 phosphate dehydrogenase and show diminished bactericidal activity after phagocytosis. The condition resembles chronic granulomatous disease in reduced myeloperoxidase activity and the susceptibility to microbial agents, but the NBT test may be normal.

Job's syndrome: This is characterised by multiple large 'cold' staphylococcal abscesses containing abundant quantities of pus, occurring repeatedly on the skin and in various organs, with little inflammatory response. Atopic eczema, chronic nasal discharge and otitis media are common features. The serum immunoglobulins are normal, except for elevated IgE. The pathogenesis of the syndrome is not clear, but probably it is a primary defect in phagocytic function.

Tuftsin deficiency: A leucokinin capable of stimulating phagocytosis, discovered at the Tufts University, Boston, has been designated 'tuftsin'. Chemically it is a small tetrapeptide (Thr-Lys-Pro-Arg). Patients with tuftsin deficiency have been reported to be prone to local and systemic bacterial infections.

Lazy leucocyte syndrome: The basic defect here is in chemotaxis and neutrophil mobility. The bone marrow has a normal number of neutrophils, but there is a peripheral neutropaenia, with poor leucocyte response to chemical and inflammatory stimulation. Patients show an increased susceptibility to bacterial infection, with recurrent stomatitis, gingivitis and otitis.

Hyper-IgE syndrome: These patients, of both sexes, have an early onset of eczema and recurrent bacterial infection such as abscesses, pneumonia and secondary infection of eczema. The organisms responsible include *Staphylococcus aureus* and *Streptococcus pyogenes*. Cellular and humoral immune mechanisms are normal, but serum IgE levels are usually more than ten times the normal level.

Actin-binding protein deficiency: Frequent infection and slow mobility of leucocytes result from defective actin-binding protein in these patients.

Shwachman's disease: In this conditon, frequent infections are found together with decreased neutrophil mobility, pancreatic malfunction and bone abnormalities.

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SECONDARY IMMUNODEFICIENCIES

A variety of factors such as malnutrition, malignancy, infections, metabolic disorders and cytotoxic drugs may lead to deficits in specific and nonspecific immunity. Secondary immunodeficiencies are therefore very much more common than primary deficiencies.

Deficiencies of humoral and cellular immune response may occur secondarily during the course of many disease processes. Humoral deficiency results when B cells are depleted as in lymphoid malignancy, particularly in chronic lymphatic leukaemia; when immunoglobulin catabolism is increased as in nephrotic syndrome, when excessive loss of serum protein occurs as in exfoliative skin disease and in protein losing enteropathies; and when excessive production of abnormal immunoglobulins occurs as in multiple myeloma. Cell mediated immunity is depressed in lymphoreticular malignancies, as in Hodgkin's disease; obstruction to lymph circulation or lymphorrhoeas; when the thymus dependent areas of lymph nodes are infiltrated with nonlymphoid cells as in lepromatous leprosy; and, transiently, following certain viral infections such as measles.

Nutritional deprivation affects both types of immune responses adversely. Ageing also causes waning in the efficiency of acquired immunity. Immunodeficiency follows the intentional or

unintentional administration of immunosuppressive agents:

Acquired immunodeficiency syndrome (AIDS), the most important of secondary immunodeficiency diseases, is considered in a separate chapter.

Further Reading

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18 Hypersensitivity

→ effect on host due to heightened or exaggerated immune response to an Ag

Immunity was originally considered a protective process, helping the body to overcome infectious agents and their toxins, but this is only one aspect of the broad phenomenon of *immunity*, which includes all manner of specific responses to antigens. Immune response may sometimes be injurious to the host. Sensitised individuals respond to subsequent antigenic stimuli in a heightened or exaggerated manner, leading to tissue damage, disease or even death. *The term hypersensitivity* refers to the injurious consequences in the sensitised host, following contact with specific antigens. In *protective processes of immunity*, the focus of attention is the antigen and what happens to it — for example, killing of a bacterium or neutralisation of a toxin. In hypersensitivity, on the other hand, antigens are of little concern and often, they are noninjurious or bland substances such as serum proteins. Hypersensitivity is concerned with what happens to the host as a result of the immune reaction.

Considerable confusion is attached to the use of the term 'allergy'. As originally used by von Pirquet, allergy meant an *altered state of reactivity to an antigen*, and included both types of immune responses, protective as well as injurious. It is still used *in* this broad sense by some. Others use the term allergy to mean all immune processes harmful to the host, such as hypersensitivity and autoimmunity. Allergy is probably most commonly used as a synonym for hypersensitivity. It is sometimes employed in a narrow sense to refer to only one type of hypersensitivity, namely, 'atopy'.

For induction of hypersensitivity reactions, the host should have had contact with the antigen (allergen). The initial contact sensitises the immune system, leading to the priming of the appropriate B or T lymphocytes. This is known as the 'sensitising' or 'priming' dose. Subsequent contact with the allergen causes manifestations of hypersensitivity. This is known as the 'shocking' dose.

Classification of hypersensitivity reactions

Hypersensitivity reactions have been classified traditionally into 'immediate' and 'delayed' types based on *the time required for a sensitised host to develop clinical reactions upon reexposure to the antigen*. The major differences between the immediate and delayed types of hypersensitivity reactions are shown in Table 18.1.

The immediate and delayed reactions are subdivided into several distinct clinical types:

Immediate hypersensitivity:

1. Anaphylaxis
2. Atopy
3. Antibody mediated cell damage
4. Arthus phenomenon
5. Serum sickness

Delayed hypersensitivity:

- Infection (tuberculin) type
- Contact dermatitis type

Coombs and Gell (1963) classified hypersensitivity reactions into four types based on the different mechanisms of pathogenesis. Their classification, now widely used, is outlined below:

TABLE 18.1

Distinguishing features of immediate and delayed types of hypersensitivity

Immediate hypersensitivity	*Delayed hypersensitivity*
1. Appears and recedes rapidly.	Appears slowly, lasts longer.
2. Induced by antigens or haptens by any route.	Induced by infection, injection of antigen intradermally or with Freund's adjuvant or by skin contact.
3. Circulating antibodies present and responsible for reaction; 'antibody mediated' reaction.	Circulating antibodies may be absent and not responsible for reaction; 'cell mediated' reaction.
4. Passive transfer possible with serum.	Cannot be transferred with serum; transfer possible with lymphocytes or transfer factor.
5. Desensitisation easy, but shortlived.	Desensitisation difficult, but longlasting.

Type I: (Anaphylactic, reagin dependent). Antibodies ('cytotropic' antibodies) are fixed on the surface of tissue cells (mast cells and basophils) in sensitised individuals. The antigen combines with the cell fixed antibody, leading to release of pharmacologically active substances (vasoactive amines) which produce the clinical reaction.

Type II: (Cytotoxic or cell stimulating). This type of reaction is initiated by antibodies that react with either antigenic components of cell or tissue elements, or with antigens or haptens which are intimately associated with cells. Cell damage occurs in the presence of complement or mononuclear cells. Combination with antibody may, in some instances, cause stimulation instead of damage. An example is the 'long acting thyroid stimulator' (LATS), an antibody against some determinant on thyroid cells, which stimulates excessive secretion of thyroid hormone. Type II reactions are intermediate between hypersensitivity and autoimmunity.

Type III (immune complex or toxic complex disease). Here the damage is caused by antigen-antibody complexes. These may precipitate in and around small blood vessels causing damage to cells secondarily, or on membranes interfering with their function. In antigen excess, soluble circulating complexes may be formed with antibodies. These may be deposited on blood vessel walls or on the basement membrane, causing local inflammation and massive complement activation.

Type IV: (Delayed or cell mediated hypersensitivity). This is one aspect of cell mediated immunity. The antigen activates specifically sensitised T lymphocytes, leading to the secretion of lymphokines. Locally, the reaction is manifested by infiltration with mononuclear cells.

The classification and some of the features of hypersensitivity reactions are shown in Table 18.2.

The four types of immunopathogenic mechanisms described above are not mutually exclusive. Any given hypersensitive reaction or other immunological process may comprise components of more than one, or all of these mechanisms. The pathology and clinical features of such immunological diseases would also be influenced by the contributions of many nonimmune body mechanisms such as inflammation, complement,

coagulation, fibrinolytic and kininogenic systems, collectively called *humoral amplification systems.* (I CC FR)

Type I reactions: Reaginic

Anaphylaxis: This is the classical immediate hypersensitivity reaction. The term anaphylaxis (*ana*—without, *phylaxis*—protection) was coined by Richet (1902) to describe his observation that dogs which had survived a sublethal injection of a toxic extract of sea anemones, were rendered highly susceptible to minute doses of the toxin given days or weeks later, instead of becoming immune to it. Theobald Smith (1902) had noticed a similar phenomenon in guinea pigs, following widely spaced injections of toxin-antitoxin mixtures. Ehrlich named this the Theobald Smith phenomenon and showed that it was independent of the toxin and antitoxin used, since the phenomenon could be induced with normal serum also.

Sensitisation is most effective when the antigen is introduced parenterally, but may occur by any route, including ingestion or inhalation. In susceptible species, very minute doses can sensitise. Antigens as well as haptens can induce anaphylaxis. There should be an interval of at least 2–3 weeks between the sensitising and shocking doses. Once sensitised, the individual remains so for long periods. The shocking dose is most effective when injected intravenously, less effective intraperitoneally or subcutaneously and least effective intradermally. The shocking antigen should be identical or immunologically closely related to the sensitising antigen. The clinical features of anaphylaxis are the same with any antigen, but are different in different species. The clinical effects are due to smooth muscle contraction and increased vascular permeability. The organs affected vary with the species. Tissues or organs predominantly involved in anaphylactic reaction are known as 'target tissues' or 'shock organs'. Other changes seen in anaphylaxis are

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TABLE 18 2
Types of hypersensitivity reactions and their features

Type of reaction	*Clinical syndrome*	*time required for manifestation*	*Mediators*
Type 1: Reaginic	1 Anaphylaxis 2 Atopy	Minutes	IgE; C. Histamine and other pharmacological agents
Type II: Cytolytic and cytotoxic	Antibody mediated damage—thrombocytopaenia, agranulocytosis, haemolytic anaemia, etc	Variable: hours to days	IgG: IgM, C
Type III: Immune complex disease	1. Arthus reaction 2 Serum sickness	Varibale: hours to days	IgG: IgM, C leucocytes
Type IV: Delayed hypersensitivity	1 Tuberculin 2 Contact dermatitis	Hours to days	T cells; lymphokines; macrophages

oedema, decreased coagulability of blood, fall in blood pressure and temperature, leucopenia and thrombocytopaenia.

There exist considerable species variations in susceptibility to anaphylaxis. Guinea pigs are highly susceptible and rats very resistant. Rabbit, dog and man are of intermediate susceptibility. Anaphylaxis can be readily induced in guinea pigs. If a small dose of egg albumin is injected intraperitoneally, followed 2–3 weeks later by a slightly larger dose of the same antigen intravenously, the guinea pig will exhibit a dramatic sequence of events. Within minutes the animal becomes irritable, sneezes, coughs, experiences respiratory distress, develops convulsions and dies. The heart continues to beat for some time after the respiration has stopped. At autopsy, the lungs are markedly emphysematous and do not collapse when the thorax is opened or even when they are cut into pieces. The shock organ is the lung. Death is due to constriction of the smooth muscles of the bronchioles causing respiratory standstill.

In the rabbit, death in anaphylactic shock is due to constriction of the pulmonary artery and its branches, leading to extreme dilatation of the right side of the heart. Respiratory movements continue after the cessation of the heart beat. In dogs, the reaction is slower and takes 1–2 hours. There is intense prostration with a profound fall of blood pressure. There is constriction of the hepatic venous system with gross engorgement of the liver. In man, fatal anaphylaxis is fortunately rare. Symptoms and signs of anaphylactic shock begin with itching of the scalp and tongue, flushing of the skin over the whole body and difficulty in breathing due to bronchial spasm. There may be nausea, vomiting, abdominal pain and diarrhoea, sometimes with blood in the stool. Acute hypotension, loss of consciousness and death follow. Human anaphylaxis, once commonly associated with heterologous serum therapy, is now seen mostly following antibiotic injections. Insect stings can also cause anaphylaxis in man. Prompt treatment with adrenalin can be lifesaving.

Adrenaline is to be administered, 0.5 ml of a 1 in 1000 solution, subcutaneously or intramuscularly, the dose being repeated upto a total of 2.0 ml over 15 minutes if necessary.

Cutaneous anaphylaxis: When a small shocking dose of an antigen is administered intradermally to a sensitised host, there will be a local 'wheal and flare' response (local anaphylaxis). The wheal is a pale, central area of puffiness due to oedema, which is surrounded by a flare caused by hyperaemia and subsequent erythema. Cutaneous anaphylaxis (skin test for Type I hypersensitivity) is useful in testing for hypersensitivity and in identifying the allergen responsible in atopic diseases. In highly sensitised individuals, even the skin test may lead to serious and even fatal reactions. *Hence a syringe loaded with adrenalin should always be kept ready whenever a skin test is performed to detect anaphylactic hypersensitivity.*

Passive cutaneous anaphylaxis (PCA): This test developed by Ovary (1952) is an extremely sensitive *in vivo* method for detection of antibodies. A small volume of the antibody is injected intradermally into a normal animal. If the antigen, along with a dye such as Evans blue, is injected intravenously 4–24 hours afterwards, there will be an immediate blueing at the site of intradermal injection due to vasodilatation and increased capillary permeability (wheal and flare reaction). PCA can be used to detect human IgG antibody which is 'heterocytotropic' (capable of fixing to cells of other species), but not IgE which is 'homocytotropic' (capable of fixing to cells of homologous species only).

Anaphylaxis in vitro: Isolated tissues, such as intestinal or uterine muscle strips from sensitised guinea pigs, held in a bath of Ringer's solution will contract vigorously on addition of the specific antigen to the bath. This is known as the Schultz-Dale phenomenon. The reaction is specific and will be elicited only by the antigen to which the animal is sensitive. Tissues from normal animals

can be passively sensitised by treatment with serum from sensitised animals.

→by circulating Ab (IgE)

Mechanism of anaphylaxis: Anaphylactic hypersensitivity can be passively transferred from a sensitive donor to a normal recipient by injection of serum. This passive sensitisation establishes that the reaction is determined by circulating antibody. Homocytotropic IgE antibody is the major antibody responsible for anaphylactic hypersensitivity. To a lesser extent, heterocytotropic IgG may be responsible.

IgG molecules are bound to surface receptors on mast cells in tissues and basophils in circulation. Following exposure to the shocking dose, the antigen molecules combine with the cell bound IgE, bridging the gap between adjacent antibody molecules. This cross linking increases the permeability of the cells to calcium ions and leads to degranulation, with release of biologically active substances contained in the granules. The manifestations of anaphylaxis are due to the pharmacological mediators, which are of two kinds — the primary mediators which are the preformed contents of mast cell and basophil granules, (histamine, serotonin, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor, heparin and various proteolytic enzymes) and the secondary mediators which are newly formed upon stimulation by mast cells, basophils and other leucocytes (slow reacting substance of anaphylaxis, prostaglandins and platelet activating factor).

Primary mediators of anaphylaxis

Histamine : This is the most important vasoactive amine in human anaphylaxis. Histamine is formed by the decarboxylation of histidine found in the granules of mast cells, basophils and in platelets. Released into the skin, histamine stimulates sensory nerves, producing burning and itching sensations. It causes vasodilatation and hyperaemia by an axon reflex (flare effect) and oedema by increasing capillary permeability (wheal effect) Histamine induces smooth muscle contraction in diverse tissues and organs, including vasculature, intestines, uterus and especially the bronchioles. It also stimulates secretions (secretogogue effect).

Serotonin (5-hydroxy tryptamine): This is a base derived by decarboxylation of tryptophan. It is found in the intestinal mucosa, brain tissue and platelets. It causes smooth muscle contraction, increased capillary permeability and vasoconstriction. It is important in anaphylaxis in rats and mice, but its role in man is uncertain.

Chemotactic factors: The eosinophil chemotactic factors of anaphylaxis (ECF-A) are acidic tetrapeptides released from mast cell granules which are strongly chemotactic for eosinophils. These probably contribute to the eosinophilia accompanying many hypersensitivity states. A high molecular weight chemotactic factor has been identified, which attracts neutrophils. *Heparin* is an acidic mucopolysaccharide. It contributes to anaphylaxis in dogs, but apparently not in man.

Secondary mediators of anaphylaxis

Slow reacting substance of anaphylaxis (SRS-A). This is so called because of the slow but sustained contraction of the smooth muscles that it causes. It is produced by leucocytes, mainly in lungs. They act on the smooth muscles of the larger blood vessels and of the bronchi and may be responsible for the prolonged respiratory distress in asthma. They are much more potent bronchoconstrictors than histamine and are not inhibited by antihistaminics.

Prostaglandins and leukotrienes: They are derived by two different pathways from arachidonic acid, which is formed from disrupted cell membranes of mast cells and other leucocytes. The lipoxygenase pathway leads to the formation of leukotrienes, while the cyclo-oxygenase pathway leads to prostaglandins and thromboxane. Prostaglandin F2a and thromboxane A2 are powerful, but

transient, bronchoconstrictors. Prostaglandin E2 is a bronchodilator. Prostaglandins also affect secretion by mucous glands, platelet adhesion, permeability and dilatation of capillaries and the pain threshold.

Platelet activating factor (PAF). PAF is a low molecular weight lipid released from basophils during immediate hypersensitivity. It causes aggregation of platelets and release of their vasoactive amines.

Other mediators of anaphylaxis

Besides the products of mast cells and other leucocytes, several other biologically active substances have been implicated in anaphylaxis. These include the anaphylatoxins released by complement activation and bradykinin and other kinins formed from plasma kininogens.

Anaphylactoid reaction: Intravenous injection of peptone, trypsin and certain other substances provokes a clinical reaction resembling anaphylactic shock. This is termed 'anaphylactoid reaction'. The clinical resemblance is due to the same chemical mediators participating in both the reactions. The only difference is that anaphylactoid shock has no immunological basis and is a nonspecific mechanism involving the activation of complement and the release of anaphylatoxins.

Atopy

The term 'atopy' (literally meaning out of place or strangeness) was introduced by Coca (1923) to refer to naturally occurring familial hypersensitivities of man, typified by hay fever and asthma. The antigens commonly involved in atopy are characteristically inhalants (e.g., pollen, house dust) or ingestants (e.g., eggs, milk). Some of them are contact allergens, to which the skin and conjunctiva may be exposed. These atopens are generally not good antigens when injected parenterally, but induce IgE antibodies, formerly termed as 'reagin' antibodies. Atopic sensitisation is developed spontaneously following natural contact with atopens. It is difficult to induce atopy artificially.

Predisposition to atopy is genetically determined. Atopy therefore runs in families. What is inherited is not sensitivity to a particular antigen, or a particular atopic syndrome, but the tendency to produce reagin antibody in unusually large quantities. All individuals are capable of forming reagin antibody in small amounts, but in atopics the reagin response is preponderant. About 10 per cent of persons have this tendency to overproduce reagin. It has been reported that bottle fed infants tend to develop atopy in later life more often than breast fed babies.

Atopic reagin is now known to be IgE antibody. Till recently, it was considered to be a unique type of antibody characterised by the following features.

1. Reagin could not be demonstrated by *in vitro* serological reactions such as precipitation or complement fixation. It is now possible to detect IgE by such sensitive *in vitro* techniques as passive haemagglutination or radio allergo-sorbent test (RAST).

2. Reagin was believed to be synthesised only in man. Injection of human serum rich in reagin into animals did not transfer atopy passively. It is now possible to induce atopic sensitivity in guinea pigs, though with difficulty.

3. Reaginic antibody has an affinity for skin cells. This is the basis of the Prausnitz-Kustner (PK) reaction, which was till recently the only method available for detecting reaginic antibody. Prausnitz and Kustner (1921) reported that if serum collected from Kustner, who had atopic hypersensitivity to certain species of cooked fish, was injected intracutaneously into Prausnitz, followed 24 hours later by an intracutaneous injection of a small quantity of the cooked fish antigen into the same site, a wheal and flare reaction occurred within a few minutes. As reaginic IgE is homocytotropic, the test has to be carried out on human skin. It has the risk of transmission of serum hepatitis.

4. Unlike other antibodies, reagin is heat sensitive and is inactivated at 56°C in 2–4 hours. Heating appears to damage the Fc part of the IgE molecule, which is necessary for fixation to cells.

5. Reagin antibody does not pass through the placenta.

Atopic sensitivity is due to an overproduction of IgE antibodies. This is often associated with a deficiency of IgA. This association had led to the suggestion that IgA deficiency may predispose to atopy. The distribution of lymphocytes capable of synthesising IgA and IgE is closely parallel, especially in the submucosa. In normal individuals, the inhalant and ingestant antigens are dealt with by IgA lining the respiratory and intestinal mucosa and therefore they do not come into contact with the potential IgE producing cells. When IgA is deficient, the antigens cause massive stimulation of IgE forming cells, leading to overproduction of reagin.

The symptoms of atopy are caused by the release of pharmacologically active substances following combination between the antigen and the cell fixed reagin. The clinical expression of atopic reactions is usually determined by the portal of entry of the antigen — conjunctivitis, rhinitis, gastrointestinal symptoms and dermatitis following exposure through the eyes, respiratory tract, intestine or skin, respectively. Sometimes the effects may be at sites remote from the portal of entry — e.g., urticaria following ingestion of the allergen.

Specific desensitisation (hyposensitisation) is often practised in the treatment of atopy. When prophylactic or therapeutic sera are to be administered as an emergency in persons who are hypersensitive, the method of desensitisation consists of giving a series of carefully graded injections of the serum, beginning with minute doses and continuing over a period of 6–8 hours. The full dose can then be administered without danger. The desensitisation is temporary and is believed to result from slow exhaustion of the store of intracellular histamine in mast cells by the repeated injections of the allergen. Desensitisation against atopens, such as pollen, can be effected by a series of weekly or biweekly injections of increasing amounts of the atopen. An alternative method is 'depot therapy' (injection of the allergen in an oil adjuvant). In this case, desensitisation is due to the production of IgG (blocking) antibodies, which by competition prevent the combination of the allergen with the cell fixed reagin antibody. Prior to desensitisation, the allergen has to be identified by skin testing with all the suspected antigens. Test kits containing common allergens are available for this purpose.

Type II reaction: Cytolytic and cytotoxic

These reactions involve the combination of IgG or IgM antibodies with antigenic determinants on the surface of cells leading to cytotoxic or cytolytic effects. Examples are lysis of red cells caused by anti-erythrocyte antibodies in autoimmune anaemias and haemolytic disease of the newborn. Alternatively, a free antigen or hapten may be absorbed on cell surfaces. Subsequent reaction of the combined antigen or hapten with its corresponding antibody leads to cell damage. Many drugs may act in this manner and cause complement mediated lysis of red cells, leucocytes and platelets. A classical example is *sedormid purpura*. When the sedative drug sedormid (now no longer used) is administered, it combines with platelets, altering their surface antigenicity. Antibodies are formed against the sedormid coated platelet antigens. When the drug is given subsequently, the antibodies attack the platelets causing thrombocytopaenic purpura. Many other drugs, including sulphonamides, thiazide diuretics, chlorpropamide and quinidine, cause such purpura.

Type III reactions: Immune complex diseases

Arthus reaction: Arthus (1903) observed that when rabbits were injected subcutaneously repeatedly with normal horse serum, the initial injections were without any local effect, but with later injections, there occurred intense local reac-

tion consisting of oedema, induration and haemorrhagic necrosis. This is known as the Arthus reaction and is a local manifestation of generalised hypersensitivity. The tissue damage is due to formation of antigen-antibody precipitates, which are deposited on the walls of blood vessels. This leads to increased vascular permeability and infiltration of the site with neutrophils. Leucocyte-platelet thrombi are formed that reduce the blood supply and lead to tissue necrosis. Arthus reaction can be passively transferred with sera containing precipitating antibodies (IgG) in high titre.

Serum sickness: This is a systemic form of Type III hypersensitivity. As originally described by von Pirquet and Schick (1905), this appeared 7–12 days following a single injection of a high concentration of foreign serum such as diphtheria antitoxin. The clinical syndrome consists of fever, lymphadenopathy, splenomegaly, arthritis, glomerulonephritis, endocarditis, vasculitis, urticarial rashes, abdominal pain, nausea and vomiting. The pathogenesis is the formation of immune complexes (consisting of the foreign serum and antibody to it that reaches high enough titres by 7–12 days), which get deposited on the endothelial lining of blood vessels in various parts of the body, causing inflammatory infiltration. The plasma concentration of complement falls due to massive complement activation and fixation by the antigen-antibody complexes. The disease is self-limited. With continued rise in antibody production, the immune complexes become larger in size and more susceptible to phagocytosis and immune elimination. When all foreign antigen is thus eliminated and free antibody appears, the symptoms clear without any sequelae. The latent period of 7–12 days is required only for serum sickness following a single injection. With subsequent injections, the disease manifests earlier. Serum sickness differs from other types of hypersensitivity reaction in that a single injection can serve both as the sensitising and shocking dose. As heterologous serum injections are not used often now, the syndrome is currently more commonly seen following injections of penicillin or other antibiotics.

Immune complexes occur in many diseases, including bacterial, viral and parasitic infections (e.g., poststreptococcal glomerulonephritis, hepatitis type B, malaria), disseminated malignancies and autoimmune conditions. The nephritis and arthritis seen in these conditions may be caused by deposition of immune complexes.

Type IV reactions: Delayed hypersensitivity

Type IV hypersensitivity reactions (delayed hypersensitivity) constitute one aspect of cell mediated immunity. These are provoked by specific antigens, evolve slowly and consist of a mixed cellular reaction involving lymphocytes and macrophages in particular. The reaction is not induced by circulating antibody, but by sensitised T cells, which, on contact with the specific antigen, release lymphokines that cause biological effects on leucocytes, macrophages and tissue cells. Delayed hypersensitivity cannot be passively transferred by serum, but can be by lymphocytes or by transfer factor. Two types of delayed hypersensitivity are recognised—tuberculin (infection) type and contact dermatitis type.

Tuberculin (infection) type: The archetype of delayed hypersensitivity is the tuberculin reaction. When a small dose of tuberculin is injected intradermally in an individual sensitised to tuberculoprotein by prior infection or immunisation, an indurated inflammatory reaction occurs at the site within 48–72 hours. In unsensitised individuals, tuberculin injection provokes no response. The tuberculin test therefore provides useful indication of the state of delayed hypersensitivity (cell mediated immunity) to the bacillus. The tuberculin test differs from the skin test for Type I hypersensitivity not only in the longer interval for appearance, but also in its morphology and histology.

Tuberculin type of hypersensitivity is developed in many infections with bacteria, fungi, viruses and parasites, especially when the

infection is subacute or chronic and the pathogen intracellular. A similar hypersensitivity is developed in allograft reaction and in many autoimmune diseases.

Contact dermatitis type: Delayed hypersensitivity sometimes results from skin contact with a variety of chemicals — metals such as nickel and chromium, simple chemicals like dyes, picryl chloride, dinitrochlorobenzene, drugs such as penicillin and toiletries. Sensitisation is particularly liable when contact is with an inflammed area of skin and when the chemical is applied in an oily base. Antibiotic ointments applied on patches of dermatitis frequently provoke sensitisation. The substances involved are in themselves not antigenic, but may acquire antigenicity on combination with skin proteins. Sensitisation requires percutaneous absorption. As most of the substances involved are fat soluble, passage along sebaceous glands may be the method of entry of the allergens.

Contact with the allergen in a sensitised individual leads to 'contact dermatitis', the lesions varying from macules and papules to vesicles that break down, leaving behind raw weeping areas typical of acute eczematous dermatitis. Hypersensitivity is detected by the 'patch test'. The allergen is applied to the skin under an adherent dressing. Sensitivity is indicated by itching, appearing in 4–5 hours, and local reaction which may vary from erythema to vesicle or blister formation, after 24–48 hours.

Shwartzman reaction

This is not an immune reaction, but rather an alteration in factors affecting intravascular coagulation. It is traditionally described along with hypersensitivity reactions because of a superficial resemblance.

Shwartzman (1928) observed that if a culture filtrate of *S. typhi* is injected intradermally in a rabbit, followed 24 hours later by the same filtrate intravenously, a haemorrhagic necrotic lesion develops at the site of the intradermal injection. The intradermal and intravenous injections need not be of the same or even related endotoxins. Culture suspensions or filtrates of a variety of bacteria will sensitise the skin to intravenous injection by an equally wide variety of cultures or filtrates. This absence of specificity and the short interval between the two doses preclude any immunological basis for the reaction.

The initial (preparatory) dose is characteristically an endotoxin. The intravenous (provocative) injection can be a variety of substances — bacterial endotoxins, antigen-antibody complexes, starch, serum, kaolin and others. The preparatory injection causes accumulation of leucocytes which condition the site by release of lysosomal enzymes damaging capillary walls. Following the provocative dose, there occurs intravascular clotting, the thrombi leading to necrosis of vessel walls and haemorrhage.

If both the injections are given intravenously, the animal dies 12–24 hours after the second dose. Autopsy shows bilateral cortical necrosis of the kidneys and patchy haemorrhagic necrosis in the liver, spleen and other organs. An essentially similar phenomenon was described by Sanarelli (1924) in experimental cholera. The reaction is therefore called the *Sanarelli-Shwartzman reaction* or the generalised Shwartzman reaction.

It has been suggested that mechanism similar to the Shwartzman reaction may operate in some clinical conditions such as the purpuric rashes of meningococcal septicaemia and the acute haemorrhagic adrenal necrosis found in overwhelming infections (Waterhouse-Friderichsen syndrome).

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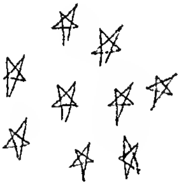

19 Autoimmunity

Self-antigens are not ordinarily immunogenic. Ehrlich (1901) observed that goats produced antibodies against erythrocytes from other goats, but not against their own, and postulated the concept of 'horror autotoxicus'. But he did not regard autoimmunisation as an impossibility and even envisaged its pathogenic possibility.

Autoimmunity is a condition in which structural or functional damage is produced by the action of immunologically competent cells or antibodies against normal components of the body. Autoimmunity literally means 'protection against self', while it actually implies 'injury to self' and therefore it has been criticised as a contradiction in terms. 'Autoallergy' has been suggested as an acceptable alternative, but the term autoimmunity has the sanction of wide usage.

The earliest example of autoimmunity was the observation by Metalnikoff (1900) that guinea pigs injected with their own spermatozoa produced sperm immobilising antibodies. Donath and Landsteiner (1904) identified circulating autoantibodies in paroxysmal cold haemoglobinuria — a haemolysin which binds with the patient's erythrocytes at low temperature and produces complement dependent haemolysis on warming. This was the first description of an autoimmune disease in man. Dameshek and Schwartz (1938) established the autoimmune basis of acute haemolytic anaemia. With the discovery of Coombs test for incomplete antibodies it became possible to demonstrate globulins bound to the surface of erythrocytes in this condition. Autoimmunisation could be induced in experimental animals by injection of self-antigens along with complete Freund's adjuvant. The use of sensitive serological techniques led to the demonstration of auto-antibodies in several diseases and even in a proportion of healthy individuals. Some auto-antibodies, such as the anti-idiotypic antibody may even be essential for the normal functioning of the immune system.

When the concept of autoimmunity came to be accepted as a pathogenic mechanism, a large number of diseases were suggested to have an autoimmune aetiology, based on the finding of auto-antibodies in the patients. This was soon recognised to be untenable as auto-antibodies could be often the result, and not the cause, of disease. Strict criteria, therefore, became necessary to differentiate diseases in which autoimmunisation was causal, from those in which it was only incidental or secondary. Such criteria were first postulated by Witebsky (1957) and have been extended by others. The following criteria have to be satisfied before establishing the autoimmune aetiology of a disease: Criteria

1. An autoimmune response, humoral, cellular or both, must be regularly associated with the disease.
2. The antigen responsible for the immune response must be identified, isolated and characterised.
3. The same antigen must induce in experimental animals immunopathological changes as in the disease.
4. Passive transfer of the disease must be possible by transfer of antibodies or sensitised lymphocytes.

Features of AID :

Diseases of autoimmune origin usually exhibit the following features:

1. An elevated level of immunoglobulins.
2. Demonstrable auto-antibodies.
3. Deposition of immunoglobulins or their derivatives at sites of election such as renal glomeruli.
4. Accumulation of lymphocytes and plasma cells at the sites of lesions
5. Temporary or lasting benefit from corticosteroid or other immunosuppressive therapy.
6. The occurrence of more than one type of autoimmune lesion in an individual.
7. A genetic predisposition towards autoimmunity.

Mechanism of autoimmunisation

Autoimmunisation can result under the following conditions:

1. Hidden or 'sequestrated' antigens may not be recognised as self-antigens. When such antigens are released into circulation, they may induce an immune response
2. Cells or tissues may undergo antigenic alteration as a result of physical, chemical or biological influences. Such altered or 'neoantigens' may elicit an immune response
3. Immunological damage may result from immune responses induced by cross reacting foreign antigens
4. Breakdown of immunological homeostasis may lead to cessation of tolerance and the emergence of forbidden clones of immunocompetent cells capable of mounting immune response against self-antigens.
5. A variety of T and B cell defects have been suggested as possible mechanisms of autoimmunity

Certain self-antigens are present in closed systems and not accessible to the immune apparatus. These are known as sequestrated antigens. An example is the lens antigen of the eye. The lens protein is enclosed in its capsule and does not circulate in the blood. Hence immunological tolerance against this antigen is not established during fetal life. When the antigen leaks out, following cataract surgery or penetrating injury, it may induce an immune response causing damage to the lens of the other eye. An example of 'sequestration in time' is seen with sperm antigens. As spermatozoa develop only with puberty, the antigen cannot induce tolerance during fetal life. Sperm antigen is not therefore recognised as self and when it enters the circulation, it is immunogenic. This is believed to be the pathogenesis of orchitis following mumps. The virus damages the basement membrane of seminiferous tubules leading to the leakage of sperms and initiation of an immune response resulting in orchitis.

Neoantigens can arise in a variety of ways. Physical agents such as irradiation can cause antigenic alteration. Photosensitivity and cold allergy may represent sensitisation to self-antigens, altered by light and cold, respectively. Several chemicals, including drugs, can combine with cells and tissues and alter their antigenic nature. Contact dermatitis, which is traditionally considered as a type of delayed hypersensitivity, can also be taken to be an autoimmune response to skin antigens altered by combination with chemical allergens. Drug induced anaemias, leucopaenias and thrombocytopaenias often have an autoimmune basis. Infectious microorganisms, particularly viruses and other intracellular pathogens, may induce alteration of cell antigens. Viral infections, such as infectious mononucleosis, are known to often precede autoimmune diseases. Bacterial enzymes also induce alteration of cell antigens. Neuraminidases formed by myxoviruses and many bacteria act on erythrocytes releasing the T antigen. The almost universal occurrence of T agglutinins in human sera is believed to represent a harmless autoimmune response following infections. Neoantigens may also arise by mutation. Such mutant cells may be immunogenic.

The fortuitous sharing of antigens by different organisms is the basis of the 'cross reacting antigen' theory of autoimmunity. Organ specific antigens are present in several species. Injection of

heterologous organ specific antigens may induce an immune response damaging the particular organ or tissue in the host. An example is the neurological injury that sometimes follows antirabic immunisation in man. Antirabies vaccine consists of infected sheep brain tissue partially denatured by treatment with phenol. Its injection elicits an immune response against sheep brain antigens. This may cause damage to the individual's nerve tissue due to the cross reaction between human and sheep brain antigens. Immunological injury due to cross reacting antigens can also follow infections. Streptococcal M proteins and heart muscle share antigenic characteristics. The immune response induced by repeated streptococcal infection can therefore damage the heart. Nephritogenic strains of streptococci possess antigens found in renal glomeruli. Infection with such strains may lead to glomerulonephritis due to the antigenic sharing. A polysaccharide antigen of *Escherichia coli* 0 14 is similar to an antigen found in the human colon. It has been suggested that ulcerative colitis in which anticolon antibodies occur may represent an immune response initiated by the cross reacting *E. coli* antigen.

Autoimmunisation may result when tolerance to a self-antigen is abrogated, as for instance by the injection of the self-antigen with Freund's adjuvant.

Enhanced helper T cell and decreased suppressor T cell functions have been suggested as causes of autoimmunity. Defects in the thymus, in stem cell development and macrophage function have also been postulated as causes. Another hypothesis is polyclonal B cell activation. While an antigen generally activates only its corresponding B cell, certain stimuli nonspecifically turn on multiple B cell clones. Such stimuli include chemicals (e.g., 2-mercaptoethanol), bacterial products (e.g., PPD, lipopolysaccharide), enzymes (e.g., trypsin), antibiotics (e.g., nystatin) and infections with some bacteria (e.g., mycoplasma), viruses (e.g., EB virus) and parasites (e.g., malaria). Multiple nonspecific antibodies form during some infectious diseases, such as antihuman erythrocyte cold antibodies in mycoplasma pneumonia and antisheep erythrocyte antibody in infectious mononucleosis. Defects in the idiotype-antidiotype network have also been said to lead to autoimmunity. Genetic factors such as defective *Ir* or immunoglobulin genes have also been postulated. In human autoimmune diseases and in animal models, genetic factors appear to influence the development and fate of autoimmune states. In spite of so many different possible mechanisms proposed, their actual role in autoimmunity, if any, has not been established.

An inbred strain of mice (New Zealand Black, NZB) has been studied extensively as a model of autoimmunity. These mice regularly develop spontaneous haemolytic anaemia and other autoimmune diseases from the age of 20 weeks.

Classification of autoimmune diseases

Based on the site of involvement and nature of lesions, autoimmune diseases may be classified as haemocytolytic, localised (or organ specific), systemic (or nonorgan specific), and transitory diseases.

HAEMOCYTOLYTIC AUTOIMMUNE DISEASES

1. Autoimmune haemolytic anaemias: Auto-antibodies against erythrocytes are demonstrable in this condition. Serologically, two groups of autoimmune anaemias can be distinguished, characterised by 'cold' and 'warm' antibodies, respectively.

The cold auto-antibodies are, generally, complete agglutinating antibodies belonging to the IgM class and agglutinate erythrocytes at 4°C, but not at 37°C. Cold agglutinins were first detected by Donath and Landsteiner in paroxysmal cold haemoglobinuria. This condition, which used to frequently accompany syphilitic infection is seldom seen nowadays. Cold agglutinins are also seen in primary atypical pneumonia, trypanosomiasis and blackwater fever.

The warm auto-antibodies are generally

incomplete, nonagglutinating antibodies belonging usually to the IgG class. They can be demonstrated coating the erythrocytes in the direct Coomb test. Warm anti-erythrocyte antibodies are frequently seen in patients taking the drug alpha methyl dopa.

In autoimmune anaemias, the red cells coated with antibodies are prematurely destroyed in the spleen and liver. Complement dependent intravascular haemolysis appears, to be a rare event.

2. *Autoimmune thrombocytopaenia:* Auto-antibodies directed against platelets occur in idiopathic thrombocytopaenic purpura. Sedormid purpura is an instance of immune response against drug induced neoantigens on platelets. This condition is traditionally considered antibody mediated hypersensitivity.

3. *Autoimmune leucopaenia.* Nonagglutinating antileucocyte antibodies can be demonstrated in the serum of patients with systemic lupus erythematosus and rheumatoid arthritis. SLE/RA

LOCALISED (ORGAN SPECIFIC) AUTOIMMUNE DISEASES

1. Autoimmune diseases of the thyroid gland

a) *Hashimoto's disease (Lymphadenoid goitre):* This is the most typical and best studied of organ specific autoimmune diseases. In 1956, Roitt and Doniach in England demonstrated antithyroglobulin antibodies in the sera of patients by precipitation in gel, and Witebsky and Rose in the U S A by the more sensitive passive haemagglutination test. The latter workers also reproduced the disease in rabbits by immunisation with autologous thyroid tissue obtained by hemithyroidectomy

Hashimoto's disease occurs more frequently in females and is associated with an enlargement of the thyroid gland and symptoms of hypothyroidism or frank myxoedema. Histologically, the glandular structure is replaced by lymphoid tissue consisting of lymphocytes, histiocytes and plasma cells. Antibodies with different specificities have been found in this condition. They include antibodies that react with thyroglobulin, a second acinar colloid, microsomal antigen and a thyroid cell surface component.

b) *Thyrotoxicosis (Graves' disease):* The majority of patients with thyrotoxicosis possess antibody to thyroglobulin. Lymphocytic infiltration is common in thyrotoxic glands. The immunological basis of thyrotoxicosis is supported by the identification of the 'long acting thyroid stimulator' (LATS) which is an IgG antibody to thyroid membrane antigen. Combination of LATS with the surface membrane of thyroid cells seems to stimulate excessive hormone secretion.

LATS – IgG Ab to thyroid membrane Ag

2. Addison's disease

Ab against cells of zona glomerulosa

The immunological basis of Addison's disease is suggested by lymphocytic infiltration of adrenal glands and the presence of circulating antibody directed against the cells of the zona glomerulosa. Similar lesions can be produced in experimental animals by immunisation with adrenal tissue in Freund's adjuvant

3. Autoimmune orchitis Ab to sperms & germinal cells

Experimental allergic orchitis with progressive damage to germinal epithelium and aspermatogenesis can be induced in guinea pigs by the injection of autogenous or allogeneic testes with Freund's adjuvant. A similar condition sometimes follows mumps orchitis. Lymphocytic infiltration of the testes and circulating antibodies to sperms and germinal cells can be demonstrated in this condition.

4. Myasthenia gravis Antimuscle Ab → ... of myoneural junction.

In this disease there is an abnormal fatiguability of muscles due to malfunction of the myoneural junction. Patients frequently have antimuscle antibodies in serum. The thymus shows lymphoid hyperplasia and numerous germinal centres. Infants born of affected mothers show symptoms

of the disease, but recover spontaneously by the age of two months, coinciding with the disappearance of maternal antibodies. This suggests that the pathogenic factor in neonatal myasthenia may be auto-antibody passively acquired from the mother.

5. Autoimmune diseases of the eye

Two types of autoimmune diseases are seen in the eye. Cataract surgery sometimes leads to intra-ocular inflammation caused by the autoimmune response to the lens protein. This is known as phacoanaphylaxis. Perforating injuries of the eye, particularly those involving the iris or ciliary bodies are often followed by sympathetic ophthalmia in the opposite eye. The disease can be produced in experimental animals by immunisation with uveal or retinal tissue in Freund's adjuvant and can be passively transferred with spleen or lymph node cells, but not with serum.

6. Pernicious anaemia

Ab — 1) parietal cells of gastric mucosa
2) intrinsic factor

Two types of auto-antibodies are present in this condition. The first is directed against the parietal cells of the gastric mucosa. This is believed to cause achlorhydria and atrophic gastritis. The second type of antibody is directed against the *intrinsic factor* and prevents absorption of vitamin B_{12} either by blocking its attachment to the gastric intrinsic factor or by binding to the B_{12}- intrinsic factor complex and interfering with its uptake by the intestinal mucosa.

7. Autoimmune diseases of the nervous system

The '*neuroparalytic accidents*' *following* rabies vaccination represent injury to the nervous system by the immune response against the sheep nervous tissue in the vaccine, which cross reacts with human nerve tissue. An essentially similar condition, experimental allergic encephalomyelitis (EAE), can be produced in animals by immunisation with nervous tissue in Freund's adjuvant.

Idiopathic polyneuritis (Guillain-Barre syndrome) is considered to be an autoimmune response against peripheral nervous tissue. It can be reproduced in experimental animals by immunisation with peripheral nervous tissue in adjuvant.

SYSTEMIC (NONORGAN SPECIFIC) AUTOIMMUNE DISEASES

This group includes conditions characterised by immune response against a variety of self-antigens and damage to several organs and tissue systems. Klemperer (1942) classified a number of diseases of unknown origin with the common feature of connective tissue lesions as 'collagen diseases'. Included in this category are systemic lupus erythematosus (SLE), rheumatoid arthritis, polyarteritis nodosa, Sjogren's syndrome, dermatomyositis and scleroderma. All these conditions are associated with generalised autoimmune processes.

1. Systemic lupus erythematosus

SLE

This is a chronic, multisystem disease with remissions and exacerbations, terminating fatally. Patients have a variety of auto-antibodies directed against cell nuclei, intracytoplasmic cell constituents, immunoglobulins, thyroid and other organ specific antigens. The main diagnostic feature is the presence of antinuclear (anti-DNA) antibody demonstrable by the LE cell phenomenon. *When the patient's blood is incubated, the* anti-DNA antibody reacts with cell nuclei, which are then phagocytosed by neutrophils. The LE cell is a neutrophil containing the phagocytosed nucleus of another cell as a large hyaline inclusion. Biological false positive reaction is seen in standard tests for syphilis. The abundance and variety of auto-antibodies suggest a breakdown in the central control of immunological homeostasis.

The clinical disease resembles the spontaneous autoimmune condition seen in NZB mice. A clinical picture resembling SLE has been observed in patients taking drugs such as diphenyl hydantoin,

isoniazid, hydralazine and procainamide. The symptoms subside on withdrawal of the drugs.

2. Rheumatoid arthritis

auto - Ab - RA (IS IgM) against Fc factor of Ig

This is a symmetric polyarthritis with muscle wasting and subcutaneous nodules, commonly associated with serositis, myocarditis, vasculitis and other disseminated lesions. It is found most commonly in adult females, though no age is exempt. The synovial membranes of the affected joints are swollen and oedematous, with dense infiltration of lymphocytes and plasma cells. A striking feature is the presence of a circulating auto-antibody called the 'rheumatoid factor' (RF). This is usually a 19S IgM, though IgG, and IgA RF have also been demonstrated. RF acts as an antibody against the *Fc* fragment of immunoglobulins. They combine usually with IgG though some types of RF are directed towards other immunoglobulin classes. RF reacts with autologous, isologous or heterologous immunoglobulins. RF is generally considered to be an immunoglobulin behaving as antibody to determinants present in the patient's own IgG molecules, though some configurational alteration of IgG may be required before its reactivity with RF becomes demonstrable.

RF is detected by agglutination tests using as antigens particles coated with globulins. In the Rose-Waaler test, the original technique for detection of RF, sheep erythrocytes coated with subagglutinating dose of anti-erythrocyte antibody (amboceptor) are used as the antigen in an agglutination test. In modifications of the test, latex and bentonite are used as the carrier particles for IgG. Antinuclear antibodies are frequently found in rheumatoid arthritis.

3. Polyarteritis nodosa

coronary thrombosis, cerebral haemorrhage, GI bleeding

This is a necrotising angiitis involving medium-sized arteries, ending fatally due to coronary thrombosis, cerebral haemorrhage or gastrointestinal bleeding. Polyarteritis is seen as a component of serum sickness and other toxic complex diseases. Immune complexes of the hepatitis antigen (HBs Ag) in affected tissues, including the kidneys, have been demonstrated in 30–40 per cent of patients. Though it has been suggested that polyarteritis nodosa may be an autoimmune disease, the auto-antibody responsible has not been idenified.

4. Sjogren's syndrome

conjunctivitis sicca, dryness of mouth, salivary gland enlargement, rheumatoid arthritis

This is a triad of conjunctivitis sicca, dryness of the mouth, with or without salivary gland enlargement, and rheumatoid arthritis. The syndrome may occur in association with other collagen diseases. Antinuclear antibodies and rheumatoid factor commonly occur in sera.

TRANSITORY AUTOIMMUNE PROCESSES

Infections or drugs — Antigenic alteration

These include conditions such as anaemia, thrombocytopaenia or nephritis that follow certain infections or drug therapy. The infecting agent or drug induces antigenic alteration in some self-antigens. The immune response set up causes tissue damage. The disease is transient and undergoes spontaneous cure when the infection is controlled or the drug withdrawn.

Pathogenesis of autoimmune disease

Many diseases are considered to be of autoimmune origin based on their association with cellular or humoral immune responses against self-antigens. Auto-antibodies are more easily detected than cellular autosensitisation. But the mere presence of auto-antibodies during the course of a disease does not prove their aetiological role. Auto-antibody formation may be a result of tissue injury and the antibody may help in promoting immune elimination of the damaged cell or tissue elements. A typical example is lepromatous leprosy in which large amounts of many auto-antibodies are regularly found. It has been said that but for the lepra bacillus, lepromatous leprosy may have been proposed as an autoimmune disease.

The relative importance of humoral and cellular immune process in the aetiology of autoimmune diseases is not known. Antibodies may cause damage by the cytolytic or cytoxic (type 2) and toxic complex (type 3) reactions. They are obviously important in haemocytolytic autoimmune diseases. A third mechanism of autoimmune tissue damage is by sensitised T lymphocytes (type 4 reaction). It is likely that humoral and cellular immune responses may act synergistically in the production of some autoimmune diseases. For example, experimental orchitis can be induced only when both types of immune responses are operative. i.e. addison's disease, graves dise

Once initiated, most autoimmune responses tend to be self-perpetuating. Their progress can be arrested by immunosuppressive therapy, though the degree of response to such therapy varies in different diseases.

Further Reading

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20 Immunology of Transplantation and Malignancy

When, as a result of disease or injury, an organ or tissue becomes irreparably damaged, or when an organ is congenitally defective or absent, a transplantation or grafting becomes necessary for restoration of function. The tissue or organ transplanted is known as the transplant or graft. The individual from whom the transplant is obtained is known as the donor and the individual on whom it is applied, the recipient.

Transplantation is one of mankind's ancient dreams. Chimaeras, fanciful creatures composed of parts from different species, figure in the mythology and pantheon of all ancient nations. But such transplantations across the species barrier do not succeed. It had been recognised very early that transplants survive only when the tissue or organ is taken from the recipient himself, while grafts from another individual of the same species or from a different species would be rejected. The earliest application of transplantation appears to have been skin grafting for reconstruction of the severed nose, using the patient's own skin flaps — a technique described in the Sushruta Samhita (Circa 800 B.C.)

The reasons for the rejection of exogenous grafts were not identified till recently. Graft rejection had for long been suspected to be due to active immunity, but it was only in the 1940s that the work of Medawar and his colleagues conclusively proved its immunological basis.

Classification of transplants

Transplants may be classified in various ways:

1. Based on the organ or tissue transplanted, they are classified as kidney, heart, skin transplant, etc.

2. Based on the anatomical site of origin of the transplant and the site of its placement, grafts are classified as 'orthotopic' and 'heterotopic'. Orthotopic grafts are applied in anatomically 'normal' sites, as in skin grafts. Heterotopic grafts are placed in anatomically 'abnormal' sites, as when thyroid tissue is transplanted in a subcutaneous pocket.

3. Transplants may be of fresh tissues and organs or of stored ones.

4. Transplants may be of living or dead materials. Live grafts, such as kidney or heart, are expected to survive and function physiologically in the recipient and are called 'vital grafts'. Nonliving transplants like bone or artery merely provide a scaffolding on which new tissue is laid by the recipient. They are called 'static' or 'structural' grafts.

5. Transplants may be classfied based on the genetic (and antigenic) relationship between the donor and the recipient (Table 20.1). An organ or tissue taken from an individual and grafted on himself is an *autograft*. A graft taken from an individual and placed on another individual of the same genetic constitution is called an *isograft*. Grafts made between identical twins or between syngenetic members of highly inbred strains of animals are examples of isografts. Grafts between two genetically nonidentical members of the same species are called *allografts* (formerly called homografts). Grafts between members of

different species are called *xenografts* (formerly called heterografts).

The allograft reaction

Same species but different genetically

When a skin graft from an animal (such as a rabbit) is applied on a genetically unrelated animal of the same species, the graft appears to be accepted initially. The graft is vascularised and seems morphologically and functionally healthy during the first two or three days. But by about the fourth day, inflammation becomes evident and the graft is invaded by lymphocytes and macrophages. The blood vessels within the graft are occluded by thrombi, the vascularity diminishes and the graft undergoes ischaemic necrosis. With extending necrosis, the graft assumes a scab-like appearance and sloughs off by the tenth day. This sequence of events resulting in the rejection of the allograft is known as the 'first set response'.

If, in an animal which has rejected a graft by the first set response, another graft from the same donor is applied, it will be rejected in an accelerated fashion. Vascularisation commences; but is soon interrupted by the inflammatory response. Necrosis sets in early and the graft sloughs off by the sixth day. The accelerated allograft rejection is known as the 'second set response'.

Mechanism of allograft rejection

The immunological basis of graft rejection is evident from the specificity of the second set response. Accelerated rejection is seen only if the second graft is from the same donor as the first. Application of a skin graft from another donor will evoke only the first set response.

An allograft will be accepted if the animal is rendered immunologically tolerant. If suitable living cells (such as splenic cells) from one pure line strain of animal is injected into fetal or neonatal animals of another inbred strain, the latter, when they grow up, will accept grafts from the former animal. This is due to the induction of 'specific immunological tolerance' against the donor tissues as a result of contact with them during embryonic life. The tolerance can be abolished by injecting lymphocytes from a non-tolerant syngeneic animal, or more effectively, from a syngeneic animal sensitised against the donor tissues by a prior allotransplantation. This method of transferring immunity by means of lymphoid cells is known as 'adoptive immunisation'.

Transplantation immunity is predominantly cell mediated. The first set response is brought about almost exclusively by T lymphocytes. Humoral antibodies are also produced during

TABLE 20.1
Terminology of grafts

Donor	Name	Synonyms
Self	Autograft	Autogenous or autogeneic graft
Genetically identical with recipient Identical twin or member of same inbred strain	Isograft	Isogeneic or syngeneic graft
Genetically unrelated member of same species	Allograft	Allogeneic graft. Formerly called Homograft
Different species	Xenograft	Xenogeneic. Formerely called Heterograft

allograft rejection. They can be detected by a variety of methods including haemagglutination, lymphocytotoxicity, complement fixation and immunofluorescence. Antibodies are formed more rapidly and abundantly during a second set response than during primary rejection. Antibodies are believed to participate in the second set response along with cell mediated immunity.

When a graft is applied to an animal possessing the specific antibodies in high titre, hyperacute rejection takes place. The graft remains pale and is rejected within hours even without an attempt at vascularisation. This is known as the 'white graft response'. This type of hyperacute rejection is sometimes seen in human recipients of kidney transplants, who may possess preexisting antibody as a result of prior transplantation, transfusion or pregnancy. The glomeruli in such cases are choked by platelet and leucocyte agglomerates.

Humoral antibodies may sometimes act in opposition to cell mediated immunity, by inhibiting graft rejection. This phenomenon, called 'immunological enhancement' was originally described by Kaliss in tumour transplants. If the recipient is pretreated with one or more injections of killed donor tissue and the transplant applied subsequently, it survives much longer than in control animals. The enhancing effect can be passively transferred to normal animals by an injection of serum from immunised animals, showing that the effect is due to humoral antibodies. The antibodies may bring about the enhancing effect in various ways. They may combine with the antigens released from the graft so that they are unable to initiate an immune response (afferent inhibition). The antibodies may combine with the lymphoid cells of appropriate specificity and, by a negative feedback influence, render them incapable of responding to the antigens of the graft (central inhibition). They may also cause 'efferent inhibition' by coating the surface of cells in the graft so that sensitised lymphocytes are kept out of contact with them.

Allograft immunity is a generalised response directed against all the antigens of the donor. A recipient sensitised by a skin graft will reject by the second set response not only another skin graft, but also any other organ or tissue graft from the same donor.

Histocompatibility antigens

Immune response against transplants depends on the presence in the grafted tissue, of antigens that are absent in the recipient and, therefore, recognised as foreign. It follows, therefore, that if the recipient possess all the antigens present in the graft, there will be no immune response, and consequently no graft rejection, even when the donor and recipient are not syngeneic. The first generation (F_1) hybrids between two inbred strains possess antigens representative of both the parent strains and will therefore accept grafts from either of the parental strains. If the two parental strains have genotypes AA and BB, respectively, the F_1 hybrid will be of genotype AB. It can therefore accept tissues with genotype AA as well as BB, as it possesses both alleles. Transplantation in the reverse direction (from F_1 to parent) will not succeed as strain AA will react against antigen B and strain BB against antigen A.

While transplants between members of a highly inbred strain of animals are successful, an exception is seen when the donor is a male and the recipient a female. Such grafts are rejected as the grafted male tissue (XY) will have antigens determined by the Y chromosome which will be absent in the female (XX) recipient. Grafts from females to males will succeed. This unilateral sex-linked histo-incompatibility is known as the Eichwald-Silmser effect.

Antigens that participate in graft rejection are called 'transplantation or histocompatibility antigens'. The blood group antigens are important in transplantation. The term 'major histocompatibility system' is applied to a system of cell antigens that exert a decisive influence on the fate of allografts. Major histocompatibility systems have been identified in different species — H2 in mice, AgB in rats, B in chickens, H1 in rabbits and DLA-

in dogs. The major histocompatibility system in man is the 'human leucocyte antigen (HL-A) system'. A description of the HLA system is presented in Chapter 15.

Factors favouring allograft survival

1. The most important factor in allograft survival is HLA compatibility. This is tested by HLA typing and tissue matching. HLA typing identifies the HLA antigens expressed on the surface of leucocytes.

Once a set of HLA compatible donors is available (commonly, siblings of the patient), the best donors among them can be chosen by tissue matching. This is done by MLR or mixed leucocyte culture (MLC). It depends on the fact that T lymphocytes in culture, when exposed to HLA incompatible antigens, will undergo blast transformation, the intensity of the reaction being a measure of the antigenic disparity between the donor and recipient lymphocytes. The test, as performed, is a one-way test in which the donor lymphocytes are killed and only the recipient lymphocytes are permitted to be transformed in response to incompatible antigens on donor cells.

2. As allograft rejection is an immunological process, immunosuppression will inhibit it. This can be achieved in experimental animals by neonatal thymectomy, chronic lymphatic drainage or administration of ALS — procedures that will inhibit cell mediated immunity. In clinical transplantation, a combination of immunosuppressive techniques is employed, including irradiation, corticosteroids, ALS and immunosuppressive drugs.

3. There appears to be certain privileged sites where allografts are permitted to survive, free from immunological attack. The fetus can be considered *an intrauterine allograft* as it contains antigens which are foreign to the mother. The reason why the fetus is exempt from rejection is not clear, though many explanations have been offered. The placenta acts as an immunological barrier by generating a hormone which is locally immunosuppressive. Major histocompatibility complex (MHC) antigens are present only in a low density on trophoblastic cells and the cell membranes are relatively resistant to attack by T or K cells. Antigen shedding by the fetus blocks aggressive T cells or antibody by an enhancement effect. An incomplete mucopolysaccharide barrier rich in sialic acid surrounds the trophoblastic cells, protecting them from cytotoxic lymphocytes.

Any site that is impenetrable to immunocompetent cells (e.g., cartilage) is a privileged site. Areas where a lymphatic drainage system is absent (e.g., brain, hamster cheek pouch) or ineffective (e.g., testes) can accept allografts without rejection. Lack of vascularity at the site also prevents graft rejection. This is the reason for the success of corneal transplants.

Graft-versus-host reaction

Graft rejection is due to the reaction of the host to the grafted tissue (host-verus-graft response). The contrary situation, in which the graft mounts an immune response against the antigens of the host, is known as the 'graft-versus-host (GVH) reaction.'

GVH reaction occurs when the following conditions are present:

1. The graft contains immunocompetent T cells.
2. The recipient possesses transplantation antigens that are absent in the graft.
3. The recipient must not reject the graft.

Examples of situations leading to GVH are:

1. Allograft in a recipient in whom specific immunological tolerance has been induced.
2. Adult lymphocytes injected into an immunologically deficient recipient. The immunological deficiency may be due to immaturity (newborn) or immunosuppression.
3. F_1 *hybrid receiving a transplant from either* parental strain.

The major clinical features of GVH reaction in animals are retardation of growth, emaciation, diarrhoea, hepatosplenomegaly, lymphoid atrophy and anaemia, terminating fatally. The syndrome has been called the 'runt disease'.

IMMUNOLOGY OF MALIGNANCY

When a cell undergoes malignant transformation, it acquires new surface antigens. It may also lose some normal antigens. This makes a tumour antigenically different from normal tissues of the host. A tumour can, therefore, be considered an allograft and be expected to induce an immune response.

Clinical evidence of immune response in malignancy

Several clinical observations indicate the presence of an immune response that prevents, arrests and occasionally cures malignancies.

1. Instances of spontaneous regression of established tumours have been reported, especially with neuroblastoma and malignant melanoma. On the analogy of the role played by the immune response in recovery from infections, it is believed that recovery from malignancy also may represent an immune process.

2. Dramatic cures sometimes follow chemotherapy of choriocarcinoma and Burkitt's lymphoma. Even a single dose of cytotoxic drug may, on occasion, result in a complete cure. Again, in some types of tumours, such as hypernephroma with pulmonary metastases, removal of the primary tumour often leads to a regression of the metastases. These observations suggest that once the large mass of tumour has been removed, mopping up operations can be effected by the immune process. The immune response appears to be effective only when the tumour is below a 'critical mass'.

3. The prevalence of certain types of cancers, observed unexpectedly at autopsy, is very much higher than their clinical incidence. This indicates that the immune system is able to deal with malignant cells as they arise and that only some of them are able to overcome the defence mechanisms and develop into clinical cancer.

4. Histological evidence of immune response against malignancy is provided by the presence of lymphocytes, plasma cells and macrophages infiltrating tumours. The cellular response resembles that seen in the allograft reaction. Tumours showing such cellular infiltration have a better prognosis than those without it.

5. If the immune system plays a natural role in preventing tumour development, a high incidence of malignancy should be expected in immune deficiency states. This is indeed so. An increased incidence of cancer, particularly of lymphoreticular malignancies, is found in congenital immunodeficiency states, as well as in patients undergoing chronic immunosuppressive therapy.

Tumour antigens

Tumour antigens are antigens that are present in malignant cells, but absent in the corresponding normal cells of the host.

Tumour specific antigens are present on the membranes of malignant cells and induce an immune response when the tumour is transplanted to syngeneic animals. Such tumour specific antigens which induce rejection of tumour transplants in immunised hosts are termed 'tumour specific transplantation antigens' (TSTA) or 'tumour associated transplantation antigens' (TATA).

In chemically induced tumours, the TSTA is tumour specific. Different tumours possess different TSTA, even though induced by the same carcinogen. In contrast, the TSTA of virus induced tumours is virus specific in that all tumours produced by one virus will possess the same antigen, even if the tumours are in different animal strains or species.

A second type of antigen is found in some tumours. These are the fetal antigens which are found in embryonic and malignant cells, but not in normal adult cells. The best known examples are alphafoetoprotein in hepatomas and the carcino-embryonic antigen found in colonic cancers. Their synthesis represents a de-differentiation of malignant cells into more primitive forms.

Carcino-embryonic antigen is a glycoprotein which can be detected in the serum of many

patients with carcinoma of the colon, particularly in the presence of metastases. But as it also appears in some other conditions such as alcoholic cirrhosis, its diagnostic value is limited. Alphafoetoprotein is an alphaglobulin secreted by normal embryonic hepatocytes. Its serum level drops sharply after birth and is hardly detectable in adults. High levels are present in hepatic carcinoma, in which condition it is of diagnostic value.

Immune response in maligancy

Both humoral and cellular responses can be demonstrated in malignancy. Anti-TSTA antibodies can be demonstrated by indirect membrane immunofluorescence. Delayed hypersensitivity to tumour antigens can be detected by skin testing with tumour cell extracts. Cell mediated immunity can be demonstrated by a stimulation of DNA synthesis and lymphokine production by the patient's leucocytes on exposure to the tumour antigens. The lymphocytes from patients are cytotoxic to cultured tumour cells.

Cell mediated immunity is believed to be the mechanism of host defence against malignancy. The humoral response may not be relevant, or may even be detrimental by facilitating tumour growth by the process of enhancement.

Immunological surveillance

The concept of immunological surveillance had its beginning in the observations of Ehrlich (1906). It was revived by Lewis Thomas in the 1950s and developed by Burnet. It postulates that the primary function of cell mediated immunity is to 'seek and destroy' malignant cells that arise by somatic mutation. Such malignant mutations are believed to occur frequently and would develop into tumours but for the constant vigilance of the immune system. Inefficiency of the surveillance mechanism, either as a result of ageing or in congenital or iatrogenic immunodeficiencies, leads to an increased incidence of cancer. While this hypothesis is attractive, it may perhaps represent an oversimplification of a complex situation.

If immunological surveillance is effective, cancer should not occur. The development of tumours represents an escape from surveillance. The mechanisms of such escape are not clear, but several possibilities have been suggested. Due to the very fast rate of proliferation of malignant cells, they may be able to 'sneak through' before the development of an effective immune response and once they reach a certain mass, may be beyond control by immunological attack. Circulating tumour antigens may act as a 'smoke screen', coating the lymphoid cells and preventing them acting on the tumour cells. The tumour antigens on malignant cells may be inaccessible for sensitised cells, being covered by some antigenically neutral substance. Humoral antibodies may cause immunological enhancement. 'Blocking' activity has been demonstrated in humoral factors. This may be due to circulating antigen, antibody or antigen-antibody complexes. Whatever be the reasons for immunological escape, tumour immunity does not appear to be a very effective mechanism judging from the high prevalence of cancer.

Immunotherapy of cancer

Different approaches have been attempted in the immunotherapy of cancer — passive, active and adoptive immunotherapy, specific and nonspecific.

Passive immunotherapy was the earliest method of cancer immunotherapy. Antisera prepared by immunising animals with tumour biopsy specimens were used for the treatment of human cancer as early as 1895. This method was found useless and, therefore, abandoned. A special type of serotherapy has recently been found beneficial in experimental tumours. Appropriate antisera that possess 'deblocking' activity *in vitro* have been found to cause regression of tumours, apparently by neutralising circulating tumour antigens and permitting sensitised lymphocytes to act on tumour cells. Monoclonal antibody to tumour antigens

may have a role as a carrier in transporting cytotoxic or radioactive drugs specifically to tumour cells.

Specific active immunotherapy by injection of tumour cell 'vaccines' was tried early this century and given up as unprofitable. The method has been modified recently by using purified tumour cell membrane antigens and by employing tumour cells treated with neuraminidase to increase their immunogenic potential.

Nonspecific active immunotherapy employs BCG and nonliving *Corynebacterium parvum*. Mathe, the leading proponent of cancer immunotherapy, has reported very good results in acute leukaemia, following combined treatment with BCG and allogeneic or authochthonous leukaemia blast cells. Intralesional BCG in malignant melanoma has been reported to induce complete remission in a high percentage of patients. It has also been used against intradermal recurrence of breast cancer following mastectomy. Dinitrochlorobenzene has been tried in the treatment of squamous and basal cell carcinoma of the skin.

Glucan, a pyran copolymer derived from microorganisms and levamisole, originally introduced as an anthelmintic, have been tried for stimulating CMI and macrophage functions.

Specific adoptive immunotherapy has been attempted with lymphocytes, transfer factor and 'immune RNA'. The donors have been persons who have been cured of their neoplasms or specifically immunised against the patient's tumour.

Immunotherapy is ineffective in the presence of a large mass of tumour cells. Its role appears to be more in getting rid of the residual malignant cells after the gross tumour has been removed. The best results in the treatment of cancer apparently follow an integrated approach to therapy, employing surgery, radiotherapy, chemotherapy and immunotherapy.

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21 Immunohaematology

Blood has held a mysterious fascination for man from the dawn of time. It was considered the essence of life and was believed to cure diverse diseases and restore youth and vitality to the aged. Blood transfusion had been attempted from very early times, but such attempts were fruitless and often fraught with disastrous consequences. Blood transfusion became scientifically feasible only after the discovery of blood groups by Landsteiner.

In his original experiment, Landsteiner (1900) cross tested serum from himself and five of his colleagues against their red blood cells. Three distinct patterns of agglutination were observed. Cells which failed to agglutinate with any of the serum samples were designated group O, while cells agglutinating in the two different patterns were called groups A and B, respectively. The fourth group AB was described later by his pupils von Decastallo and Sturli (1902).

The ABO system is the most important of all the blood group systems and its discovery made blood transfusion possible. No other blood group antigens were discovered for the next 25 years. Using rabbit antisera to different samples of human red cells, Landsteiner and Levine (1926) discovered the MN and P antigens. Landsteiner and Wiener (1940) raised rabbit and guinea pig antisera against Rhesus monkey erythrocytes and tested them against human red cells. This led to the discovery of the 'Rhesus (Rh) factor'. Many more blood group antigens have been identified subsequently, mostly by studying antibodies in patients who had received multiple blood transfusions or mothers of infants with haemolytic disease. The main blood group systems with the dates of their discovery are shown below.

ABO	1900	Duffy	1950
MN	1926	Kidd	1951
P	1926	Diego	1955
Rh	1940	Yt	1956
Lutheran	1945	Kg	1962
Lewis	1946	Dombrock	1965
Kell	1946	Colton	1967

Some antigens have been identified that occur only very rarely, being limited to certain individuals or families. These have been termed 'private antigens'.

ABO blood group system

The ABO system contains four blood groups and is determined by the presence or absence of two distinct antigens A and B on the surface of erythrocytes. Red cells of group A carry antigen A, cells of group B antigen B and cells of group AB have both A and B antigens, while group O cells have neither A nor B antigen. The four groups are also distinguished by the presence or absence of two distinct isoantibodies in the serum. The serum contains the isoantibodies specific for the antigen that is *absent* on the red cell. The serum of a group A individual has anti-B antibody, group B has anti-A and group O both anti-A and anti-B, while in group AB both anti-A and anti-B are absent (Table 21.1).

Group A is subdivided into A_1 and A_2. Antiserum of group A agglutinates group A_1 cells powerfully, but A_2 cells only weakly. About 80

90%

per cent of group A bloods are A_1 and 20 per cent A_2. The subgroups of A antigen are represented in group AB also. The recognition of group A subgroups increases the number of ABO phenotypes from four to six: A_1, A_2, B, A_2 B, A_1 B and O. Other A subgroups (A_3, A_4, A_5) have also been described, but they are not clinically relevant.

Blood group antigens are inherited according to simple Mendelian laws. Their synthesis is determined by allelomorphic genes, A, B and O. Genes A and B give rise to the corresponding antigens, but O is an amorph and does not produce any antigen. The frequency of ABO distribution differs in different peoples. Group O is the commonest group and AB the rarest. The ABO distribution in Britain is approximately O–47 per cent, A–42 per cent, B–8 per cent and AB–3 per cent. In India, the distribution is approximately O–40 per cent, A–22 per cent, B–33 per cent and AB–5 per cent.

Anti-A and anti-B isoantibodies appear in the serum of infants by about the age of six months and persist thereafter. These are called 'natural' antibodies because they seem to arise presumably under genetic control without any apparent antigenic stimulation. But it is likely that they develop as a result of unidentified environmental stimuli with the blood group-like antigens present in bacteria or other sources. Natural anti-A and anti-B antibodies are saline agglutinating antibodies reacting optimally between 4°C and 18°C but active also at 37°C. Immune isoantibodies may develop following ABO incompatible pregnancy or transfusion. More commonly they result from the injection of substances containing blood group-like antigens, such as horse serum or bacterial vaccines made from media containing horse or hog extracts. Immune isoantibodies are 'albumin agglutinating' antibodies, reacting optimally at 37°C, and acting as haemolysins in the presence of complement. They are important clinically as they may cause severe transfusion reactions.

H antigen: Red cells of all ABO groups possess a common antigen, the H antigen, which is a precursor for the formation of A and B antigens. The amount of H antigen is related to the ABO group of the cell, group O cells having the most and AB the least amount. Due to its universal distribution, H antigen is not ordinarily important in grouping or blood transfusion. Bhende *et al* (1952) from Bombay reported the very rare instance in which A and B antigens as well as the H antigens are absent from red cells. This is known as the 'Bombay' or OH blood. Such individuals will have *anti-A*, *anti-B* and *anti-H* antibodies, so that their sera will be incompatible with all red cells except of those with the same rare blood group.

A, B and H antigens are glycoproteins. They are not confined to the erythrocytes, but can be detected in almost all the tissues and fluids of the body. While these antigens are always present in tissues, they are found in secretions (saliva, gastric juice, sweat) of only about 75 per cent of all

TABLE 21.1

Distribution of ABO antigens and antibodies in red cells and serum

Group	Red Cells		Serum	
	Antigen present	Agglutinated by serum of group	Antibody present	Agglutinates cells of group
A	A	B,O	anti-B	B,AB
B	B	A,O	anti-A	A,AB
AB	A & B	A,B,O	None	None
O	None	None	anti-A and anti-B	A,B AB

persons. Such persons are called 'secretors' and those who lack blood group antigens in secretions are called 'non-secretors'. The secretion of ABH antigens is controlled by two allelic genes Se and se. Individuals homozygous or heterozygous for Se are secretors, while those who are se-se are non-secretors.

A and B antigens are also found in certain animals and plants. They have been extracted and purified commercially from the stomachs of horses and hogs. Blood group antibodies are also found in some animals. Substances specifically agglutinating A or B antigens have been detected in some plants. A potent anti-A_1 agglutinin has been extracted from *Dolichos biflorus* and anti-H from *Ulex europaeus*. Blood group agglutinins of plant origin are known as 'lectins'.

Rh blood group system

Levine and Stetson (1939) demonstrated a new type of antibody in the serum of a woman who developed severe reactions following transfusion of her husband's ABO compatible blood. She had recently delivered a stillborn infant with haemolytic disease. They suggested that the woman may have been sensitised by some antigen inherited by the fetus from its father. Landsteiner and Wiener (1940) identified in the red cells of the majority of persons tested, an antigen that reacted with rabbit antiserum to Rhesus monkey erythrocytes. This antigen was called the 'Rhesus' or Rh factor. The 'new type' of antibody described by Levine and Stetson was identified as anti-Rh factor antibody. Wiener and Peters (1940) demonstrated anti-Rh antibody in some persons who had received ABO compatible transfusion. Levine and colleagues (1941) proved that Rh sensitisation was the cause of haemolytic disease of the newborn.

The Rh system is complex and its study is complicated by the existence of two different theories and nomenclatures for the genes and antigens. Wiener proposed that Rh antigens are determined by any one of several allelic genes which may appear at a single locus and govern the production of the appropriate agglutinogen on the surface of erythrocytes. Each agglutinogen is in turn made up of one or more antigens. Fisher, on the other hand, postulated that Rh antigens are determined by three pairs of closely linked allelomorphic genes, *Cc*, *Dd* and *Ee*. Every individual possesses one member of each pair of these genes derived from each parent. Each gene would be responsible for the production of a specific antigen, which could be detected by its specific antibody.

The designation employed by the two systems for the different Rh types are as follows:

	Fisher	Wiener
Rh positive	CDe	Rh1
	cDE	Rh2
	cDe	Rho
	CDE	Rhz
Rh negative	Cde	rh'
	cdE	rh"
	cde	rh
	CdE	rhy

For routine purposes, the typing of persons as Rh positive or negative depends on the presence or absence of antigen D (Rho) on red cells and hence can be accomplished by testing with anti-D (anti-Rh) serum. This is because D is the most powerful Rh antigen and accounts for the vast majority of Rh incompatability reactions. The distribution of Rh positives differs in different races. Among people of European descent, about 85 per cent are Rh positive and 15 per cent negative. Among Indians, approximately 93 per cent are Rh positive and 7 per cent negative.

A variant of D is known as D^u. Red cells of D^u subtype react with some, but not with all anti-D sera. Though ^{u} cells may not be agglutinated by anti-D sera, they absorb the antibody on their surface. D^u subtype can therefore be detected by reacting red cell with anti-D serum and then doing a direct Coombs test. For purposes of blood dona-

tion, D^u cells are considered Rh positive. But when a D^u individual requires transfusion, it is advisable to use Rh negative blood because he is capable of being immunised by standard Rh positive blood.

There are no natural anti-Rh antibodies in the serum. They arise only as a result of Rh incompatible pregnancy or transfusion.

Other blood group systems

The Lewis blood group system consists of two antigens Le^a and Le^b. It differs from other blood group systems in that the antigens are present primarily in the plasma and saliva. Red cells acquire the antigen by adsorbing them from plasma. The Lewis phenotypes are closely related to the ABO group and to the secretor status of an individual. Naturally occurring Lewis antibodies are frequently found in the sera of persons lacking the corresponding antigen.

In the MN system, using rabbit antisera, persons were originally classified into three groups, M, N and MN. An antigen, S, was later added to this system. This has enabled the identification of ten groups in the MNS system.

Blood group systems other than ABO and Rh are of little clinical importance as they do not usually cause transfusion reactions or haemolytic disease. They have applications in genetics, anthropology, tissue typing and forensic medicine. As blood group antigens are inherited from the parents, they are often useful in settling cases of disputed paternity.

Medical applications of blood groups

Blood transfusion. The existence of several different blood group antigens makes it almost impossible to obtain a perfectly matched blood for transfusion. But in routine transfusion practice, only the ABO and Rh antigens are relevant. The other antigens are too weak to be of importance. Safety in blood transfusion requires that the following conditions be satisfied in choosing a donor.

1. The recipient's plasma should not contain any antibody that will damage the donor's erythrocytes.
2. The donor plasma should not have any antibody that will damage the recipient's red cells.
3. The donor red cells should not have any antigen that is lacking in the recipient. If the transfused cells posses a 'foreign antigen', it will stimulate an immune response in the recipient.

Ideally, the donor and recipient should belong to the same ABO group. It used to be held that O group cells could be transfused to recipients of any group as they possessed neither A nor B antigen. Hence O group was designated as the 'universal donor'. The anti-A and anti-B antibodies in the transfused O blood group do not ordinarily cause any damage to the red cells of A or B group recipients because they will be rendered ineffective by dilution in the recipient's plasma. But some O group plasma may contain isoantibodies in high titre (1:200 or above) so that damage to recipient cells may result. This is known as the 'dangerous O group'. The anti-A antibody in O group blood is generally more potent than the anti-B. Hence O blood group is more likely to cause reaction when given to A group recipients than to those of B group. While O group blood with low titre antibodies may be transfused to a patient of any other group in dire emergency, this practice should never be employed as a routine. Transfusion of large quantities of O group blood to persons of any other group may cause reactions.

Due to the absence of isoantibodies in plasma, AB group persons were designated 'universal recipients'. AB group donors may not always be available due to their rarity and it may, on occasion, be necessary to use donors of other groups. In such cases, group A blood is safer than group B, because anti-A antibody is usually more potent that anti-B.

Rh compatibility is important only when the recipient is Rh negative. An Rh positive person may safely receive either Rh positive or negative blood. But an Rh negative individual receiving Rh positive blood may form antibodies against the Rh antigen. A subsequent transfusion with

Rh positive blood may then cause reaction. An additional risk in women is Rh sensitisation leading to haemolytic disease of the newborn. Therefore it is particularly important that Rh negative women who are not past the childbearing age receive only Rh negative blood.

Besides ABO grouping and Rh typing of the donor and recipient, it is invariably necessary before transfusion to perform a 'cross matching' to ensure that the donor cells are compatible with the recipient plasma. Two procedures have been recommended for cross matching. In the saline cross match, washed red cells from the donor are mixed with recipient serum in a test tube and left at room temperature for two hours The sedimented cells are then examined for agglutination under the lower power of a microscope. A more satisfactory method is to incubate washed donor cells and recipient serum in a water bath at 37°C for two hours and then do a direct Coombs test. This would bring to light incomplete antibodies also, that may sometimes be responsible for transfusinn reactions.

Following an incompatible hlood transfusion, the red cells may undergo clumping and intravascular haemolysis or they may be coated by antibody, engulfed by phagocytes, removed from circulation and subjected to extravascular lysis. Incompatible transfusion may be accompanied by clinical features such as shivering, tingling sensation, bursting headache, constricting precordial discomfort and severe lumbar pain. Hypotension, cold clammy skin, cyanosis, feeble pulse and other signs of collapse may be seen. Jaundice, haematuria, oliguira and anuria may follow.

Some transfusion reactions may be due to immunological processes other than blood group incompatibility. Rigor, urticaria and other manifestations often occur due to the recipient being hypersensitive to some allergen present in the donor hlood. Serious reactions follow when haemolysed or contaminated blood is transfused. Whenever any reaction occurs, the transfusion should be stopped immediately. The remainder of the donor blood should be sent to the blood bank for investigation.

The most common complications following blood transfusion are of infectious origin. Transfusion of blood contaminated by bacteria may lead to endotoxic shock or septicaemia. Gross contamination can be recognised in most cases by inspection of the blood before transfusion, as haemolysis is usually apparent. Such contamination can be eliminated by proper techniques of blood collection and storage. Other infectious complications are due to microorganisms present in the donor The chief of these is viral hepatitis. Screening of donors for HBsAg has helped to check type B hepatitis, but it is of no avail against non-A non-B hepatitis. The most serious disease transmitted by transfusion is AIDS. Screening of donors for anti–HIV antibody can minimise the risk of infection but not eliminate it altogether as HIV viraemia may be present in infected donors even before anti-HIV antibodies appear.

Cytomegalovirus transmitted by transfusinn may cause an infectious mononucleosis-like syndrome. Syphilis may be transmitted by transfusion of fresh blood from an infectious donor, but not if the blood has been stored for two days or more before transfusion Malaria is another disease transmissible by transfusion.

Whole blood transfusion is now being replaced increasingly by blood component therapy, which causes fewer complications and results in more optimal utilisation of human hlood which is a scarce commodity. For example, in anaemia, packed red cell transfusion is more beneficial than whole hlood as it provides greater oxygen carrying capacity with less circulatory overload and minimal electrolyte disturbance Frozen red cells are available for transfusion in patients with rare blood groups. Similarly, leucocyte and platelet concentrates are available for specific needs. Plasma cryoprecipitates and Factor VIII are routinely used in haemophilia. Other coagulation factors are also available for different coagulation disorders. Such plasma products are manufactured from pooled human blood and may transmit HIV, Hepatitis type B virus and other infectious agents unless great care is taken in selection of donors and in manufacturing processes.

Almost all adverse reactions of transfusion can be eliminated by autologous blood transfusion which is rapidly becoming popular. Here, blood is collected from the individual himself and stored for use during elective surgery. Blood collection may be started a month before the expected date of transfusion. Autologous transfusion eliminates not only infectious complications, but also those due to minor blood group incompatibilities and hypersensitivity.

Haemolytic disease of the newborn

When an Rh negative woman carries an Rh positive fetus, she may be immunised against the Rh antigen by the passage of fetal red cells into the maternal circulation. Minor transplacental leaks may occur any time during pregnancy, but it is during delivery that fetal cells enter the maternal circulation in large numbers. Therefore, the mother is usually immunised only at the first delivery and, consequently, the first child escapes damage (except where the woman had been sensitised already by prior Rh incompatible transfusion). During a subsequent pregnancy, Rh antibodies of the IgG class pass from the mother to the fetus and damage its erythrocytes. This is the pathogenesis of the haemolytic disease of the newborn. The clinical features may vary from a mere accentuation of the physiological jaundice in the newborn to erythroblastosis foetalis, or intrauterine death due to hydrops foetalis.

Haemolytic disease does not affect all issues of Rh incompatible marriages. Its incidence is much less than the expected figures. The following factors influence the incidence of haemolytic disease due to Rh incompatibility:

1. *Immunological unresponsiveness to the Rh antigen*: Not every Rh negative individual forms Rh antibodies following antigenic stimulation. Some fail to do so even after repeated injections of Rh positive cells. They are called 'nonresponders'. The reason for this immunological unresponsiveness is not known.

2. *Fetomaternal ABO incompatibility*: Rh immunisation is more likely to result when the mother and fetus possess the same ABO group. When Rh and ABO incompatibility coexist, Rh sensitisation in the mother is rare. In this situation the fetal cells entering the maternal circulation are believed to be destroyed rapidly by the ABO antibodies before they can induce Rh antibodies.

3. *Number of pregnancies*: The first child usually escapes disease because sensitisation occurs only during its delivery. The risk to the infant increases with each successive pregnancy.

4. *Zygosity of the father*: An individual may be homozygous or heterozygous in respect of D antigen. When the father is homozygous all his children will be Rh positive. When he is heterozygous half his children would be Rh positive.

Detection of Rh antibodies

Most Rh antibodies are of the IgG class and being 'incomplete antibodies', do not agglutinate Rh positive cells in saline. A minority are complete (saline agglutinating) antibodies of the IgM class. These are not relevant in the pathogenesis of haemolytic disease as they do not traverse the placenta.

IgG anti-D antibodies may be detected by the following techniques: 1) using a colloid medium such as 20 per cent bovine serum albumin, 2) using red cells treated with enzymes such as trypsin, pepsin, ficin or bromelin, and 3) by the indirect Coombs test. The last is the most sensitive method.

Identification of Rh incompatibility

Rh typing should form a part of routine antenatal examination. When the woman is Rh negative, and her husband Rh positive, fetal complications should be expected. Women with Rh incompatible pregnancy should be screened for Rh antibodies by the indirect Coombs test at 32 – 34 weeks of pregnancy and at monthly intervals

thereafter. The appearance of Rh antibodies during pregnancy or their increase in titre if they were present already, would prove that the fetus is Rh positive. If amniocentesis is indicated, demonstration of Rh antigen in the amniotic fluid would also prove that the fetus is Rh positive. In the case of haemolytic disease of the newborn, the maternal serum will show Rh antibodies in the indirect Coombs test and the infant's red cells will give a positive direct Coombs test.

When haemolytic disease is diagnosed antepartum, an intrauterine transfusion with Rh negative blood may be indicated. Red cells introduced into the fetal peritoneal cavity will find their way into the circulation and will survive normally. Premature delivery followed by transfusion may be necessary in some cases. When a baby is born with haemolytic disease, exchange transfusion with Rh negative ABO compatible blood is the treatment of choice.

Prevention of Rh Isoimmunisation

Remarkable success has been achieved recently in the prevention of Rh isoimmunisation by the administration of anti-Rh IgG antibody at the time when the antigenic stimulation is expected to take place. The passively administered antibody may prevent isoimmunisation by a negative feedback mechanism or by afferent inhibition. The recommended practice is to inject 100–300 μg of Rh immune IgG to an Rh negative woman immediately after delivery. To be effective, this should be employed from the first delivery onwards. The Rh immuneglobulin for the purpose is prepared from human volunteers.

ABO haemolytic disease

Maternofetal ABO incompatibility is very common and in a proportion of these, haemolytic disease occurs in the newborn. In persons of blood group A or B, natural antibodies are IgM in nature and so do not cross the placenta to harm the fetus. But in persons of blood group O, the isoantibodies are predominantly IgG in nature. Hence ABO haemolytic disease is seen largely in O group mothers, bearing A or B group fetus. As ABO haemolytic disease is due to naturally occurring maternal isoantibodies, it may occur even in the firstborn, without prior immunisation. ABO haemolytic disease is much milder than Rh disease, probably because erythrocytes of the newborn have fewer A or B antigenic sites as compared to adult erythrocytes. The direct Coombs test is therefore often negative in this condition, while the indirect Coombs test (neonatal serum with type specific adult erythrocytes) is more commonly positive. Peripheral blood smear characteristically shows spherocytosis.

Blood group and diseases

It has been shown that some diseases may influence blood group antigens. Blood group antigens have been reported to become weak in leukaemia. The reason for this is not known. The acquisition of B antigen by Group A persons has been observed following some infections. The antigen is believed to come from the infecting microorganism.

Red cell suspensions contaminated with certain bacteria, such as *Pseudomonas aeruginosa*, become agglutinable by all blood group sera and even by normal human sera. This phenomenon, known as the Thomsen-Freidenreich phenomenon, is due to the unmasking of a hidden antigen normally present on all human erythrocytes. This is called the T antigen. Anti-T agglutinins are normally present in human sera. Such panagglutinability of red cells has occasionally been observed in persons suffering from systemic bacterial infections.

Several investigators have attempted to correlate blood group and susceptibility to certain diseases. It has been shown that duodenal ulcer is more frequent in persons of blood group O than in others. An association has also been established between group A and cancer of the stomach.

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22 Staphylococcus

Staphylococci are Gram positive cocci that occur in grape-like clusters. They are ubiquitous and form the commonest cause of localised suppurative lesions in man. Their ability to develop resistance to penicillin and other antibiotics enhances their importance as a human pathogen, especially in the hospital environment.

Staphylococci were first observed in human pyogenic lesions by von Recklinghausen in 1871. Pasteur (1880) obtained liquid cultures of the cocci from pus and produced abscesses by inoculating them into rabbits. It was Sir Alexander Ogston, a Scottish surgeon, who established conclusively the causative role of the coccus in abscesses and other suppurative lesions (1880). He also gave it the name Staphylococcus (*staphyle*, meaning a bunch of grapes, *kokkos*, meaning a berry) from the typical occurence of the cocci in the grape-like clusters in pus and in cultures. Ogston noticed that nonvirulent staphylococci were also often present on skin surfaces. Most staphylococcal strains from pyogenic lesions were found to produce golden yellow colonies and strains from normal skin, white colonies on solid media. Rosenbach (1884) named them *Staph. aureus* and *albus*, respectively. Passet (1885) described a third variety, *Staph. citreus*, producing lemon yellow colonies.

The association between virulence and pigment production was not found to be constant. As distinction between pathogenic and nonpathogenic staphylococci is important in diagnostic practice, several attempts have been made to establish a correlation between virulence and laboratory criteria that could be tested conveniently. *These included* haemolysis, gelatin liquefaction, lipolytic activity and production of urease and phosphatase. None of these was found reliable. There is a general agreement between virulence and production of the enzyme coagulase, and to a less extent, fermentation of mannitol. Staphylococci are therefore classified into two groups, based on coagulase production. *Staph. aureus* (often called *Staph. pyogenes* also) which is coagulase positive, mannitol fermenting and usually pathogenic. They produce toxins. Most strains form golden yellow colonies though some may be white or cream coloured; and *Staph. epidermidis* (often called *Staph. albus* also) which is coagulase negative, mannitol nonfermenting and usually nonpathogenic. They do not produce toxins. Most strains form white colonies, though some may be pigmented.

Staphylococcus aureus

Morphology: They are spherical cocci, approximately 1μ in diameter, arranged characteristically in grape-like clusters (Fig. 22.1). Cluster formation is due to cells dividing sequentially in three perpendicular planes and the daughter cells getting located by the action of separation enzymes to yield typical irregular clusters. They may also be found singly, in pairs and in short chains of three or four cells, especially when examined from liquid cultures. Long chains never occur. They are nonmotile, nonsporing and noncapsulated, though capsules have been demonstrated in young cultures of many strains.

They stain readily with aniline dyes and are uniformly Gram positive. Under the influence of penicillin and certain chemicals, they may change to L forms. The genus *Staphylococcus* has at least 20 species.

Fig. 22.1 Staphylococcus in a smear of pus

Cultural characteristics They grow readily on ordinary media within a temperature range of 10–42°C, the optimum temperature being 37°C and pH 7.4–7.6. They are aerobes and facultative anaerobes.

On nutrient agar, after incubation for 24 hours, the colonies are large (2–4 mm diameter), circular, convex, smooth, shiny, opaque and easily emulsifiable. Most strains produce golden yellow pigment, though some may be white, orange or yellow. The pigment does not diffuse into the medium. Pigment production occurs optimally at 22°C and only in aerobic cultures. Pigment production is enhanced when 1% glycerol monoacetate or milk is incorporated in the medium. The pigment is believed to be a lipoprotein allied to carotene.

On nutrient agar slope, their confluent growth presents a characteristic 'oil-paint' appearance.

The colonies on blood agar are similar to those on nutrient agar. Most strains are haemolytic, especially when incubated under 20–25 per cent carbon dioxide. They produce a β type of haemolysis. Haemolysis is marked on rabbit or sheep blood and weak on horse blood agar.

They grow on MacConkey's medium, producing smaller colonies that are pink due to lactose fermentation.

In liquid media uniform turbidity is produced.

Several selective media have been devised for isolating *Staph. aureus* from specimens such as faeces containing other bacteria. These include media containing 8–10 per cent NaCl (salt-milk agar, salt broth), lithium chloride and tellurite (Ludlam's medium) and polymyxin. For primary isolation sheep blood agar is recommended. Human blood should not be used as it may contain antibodies or other inhibitors.

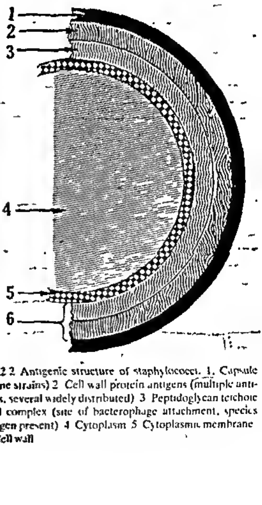

Fig. 22.2 Antigenic structure of staphylococci. 1. Capsule (some strains) 2. Cell wall protein antigens (multiple antigens, several widely distributed) 3. Peptidoglycan teichoic acid complex (site of bacteriophage attachment, species antigen present) 4. Cytoplasm 5. Cytoplasmic membrane 6. Cell wall

Biochemical reactions: They ferment a number of sugars, producing acid, but no gas. This is of no diagnostic value except for mannitol, which is usually fermented by *Staph. aureus* but not by *Staph. epidermidis.* They are catalase positive (unlike streptococci) and usually hydrolyse urea, reduce nitrates to nitrites, liquefy gelatin and are MR and VP positive, but indole negative. Most strains are lipolytic and when grown on media containing egg yolk, produce a dense opacity. Production of phosphatase can be demonstrated by culturing on nutrient agar containing phenolphthalein diphosphate. When such a culture is exposed to ammonia vapour, colonies assume a bright pink colour due to the presence of free phenolphthalein. This is a useful screening procedure for differentiating *Staph. aureus* from *Staph. epidermidis* in mixed cultures, as the former gives prompt phosphatase reaction while the latter is usually negative or only weakly positive.

The characteristics of a pathogenic strain of staphylococcus are: 1) coagulase positive, 2) greater biochemical activity, ferments mannite, 3) produces a β type of haemolysis on blood agar, 4) usually produces a golden yellow pigment, 5) liquefies gelatin, 6) phosphatase is produced, and 7) in a medium containing potassium tellurite, reduces tellurite, producing black colonies.

Resistance: Staphylococci are among the more resistant of nonsporing bacteria. Dried on threads, they retain their viability for 3–6 months. They have been isolated from dried pus after 2–3 months. They may withstand 60°C for 30 minutes. Their thermal death point is 62°C for 30 minutes. Some staphylococci require 80°C for one hour to be killed. Heat resistant strains have the ability to grow at a higher temperature, even at 45°C. Some strains grow in the presence of 10% NaCl and some others even in the presence of 15% NaCl. These have significance in food preservation.

They resist 1% phenol for 15 minutes. Mercury perchloride 1% solution kills them in 10 minutes. Many aniline dyes are strongly bactericidal, crystal violet being lethal at a concentration of one in five hundred thousand and brilliant green, one in ten million.

Fatty acids inhibit the growth of staphylococci, the highly unsaturated acids having a more powerful action on coagulase positive than on coagulase negative strains. Staphylococci are uniformly resistant to lysozyme, but some micrococci are sensitive to it. Staphylococci are generally sensitive to *Lysostaphin* — a mixture of enzymes produced by a particular strain of *Staph. epidermidis.*

Staphylococci were originally sensitive to sulphonamides, penicillin and other antibiotics, but they develop drug resistance so readily that most strains, especially those from the hospital environment, are now resistant to the drugs in common use. Resistance may be due to the following factors: 1) Beta-lactamase production, e.g., penicillin G, ampicillin, ticarcillin and similar drugs. This is plasmid controlled and the plasmids are transmitted by transduction and also possibly by conjugation. 2) Genes in the chromosome which are variably expressed, e.g., resistance to methicillin, oxacillin, nafcillin. 3) When there is difference between minimum inhibitory concentration and minimum lethal concentration, the organisms become 'tolerant'. 4) Multiple drug resistance due usually to several drug resistant genes in a single plasmid, each with its own resistance markers. A bacterial cell may carry more than one plasmid each with its own resistance marker. Plasmids carrying resistance to erythromycin, mercury and other metallic ions, tetracycline, chloramphenicol, neomycin and others have also been detected.

When the presence of a particular antibiotic is removed from the environment, they become sensitive to it.

Some strains of *Staph. aureus* carry plasmids coding for the production of bacteriocin (staphylococcin). This property appears to be limited to strains of phage group II. Staphylococcin is thermostable and is active against many Gram positive bacteria including staphylococci, streptococci, pneumococci, corynebacteria and aerobic sporing bacilli. Gram negative bacteria are resistant.

Antigenic structure

Staphylococci contain proteins, antigenic polysaccharides as well as other substances important in cell wall structure (Fig 22.2). Capsular antigens are found on mucoid untypable strains that lack bound coagulase. Peptidoglycan is a polysaccharide polymer which provides a rigid exoskeleton to the cell wall. It is destroyed by strong acid as well as by lysozyme. It elicits production of interleukin-1 (endogenous pyrogen) and opsonic antibodies by monocytes. It is a chemo-attractant for polymorphonuclear leucocytes. It has endotoxic activity, produces a localised Schwartzman phenomenon and activates complement. Teichoic acids are linked to the peptidoglycan and can be antigenic. Patients with active endocarditis due to *Staph. aureus* may have antibodies to teichoic acids detectable by gel diffusion.

Protein A is a cell wall component of many *Staph. aureus* strains. It binds to the *Fc* portion of IgG molecules except IgG3. The *Fab* portion of IgG bound to protein A is free to combine with a specific antigen. Protein A with attached IgG molecules directed against a specific bacterial antigen will agglutinate bacteria that have that antigen. This is called *coagglutination*. Protein A has thus important diagnostic uses.

Serologic tests have limited utility in identifying staphylococci.

Bacteriophage typing

Staphylococci may be typed, based on their susceptibility to bacteriophages. An internationally accepted set of phages is used for typing. Staphylococcal phage typing is by a pattern method. The strain to be typed is inoculated on a plate of nutrient agar to produce a lawn culture. After drying, the phages are applied over marked squares in a fixed dose (routine test dose). After overnight incubation, the culture will be observed to be lysed by some phages but not by others. The phage type of the strain is expressed by the designations of the phages that lyse it. Thus, if a strain is lysed only by phages 52, 79 and 80, it is called phage type 52/79/80. Phage typing is of great importance in epidemiological studies of staphylococcal infections.

The reference centre for staphylococcal phage typing in India is located in the Department of Microbiology, Maulana Azad Medical College, New Delhi. According to the information available from the centre, phage type 52/52A/80/81 is prevalent in most parts of India.

Typing set of staphylococcal phages

Group I	29, 52, 52A, 79, 80
Group II	3A, 3B, 3C, 55, 71
Group III	6, 7, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Group IV	42D
Not allocated	81, 94, 95, 96

Not all cultures are typable by this procedure, and the susceptibility patterns of circulating strains vary in time and locality. Hence phages in the reference set require periodic revisions.

Toxins and other virulence factors

Staph. aureus produces a number of exotoxins. Many toxic effects can be demonstrated using culture filtrates. Based on their biological activity, staphylococcal toxins may be classified into three groups: cytolytic toxins consisting of haemolysins and leucocidins, enterotoxin and exfoliative toxin. They also produce nontoxic aggressins such as coagulase, which contribute to pathogenicity.

Haemolysins: Four antigenically distinct haemolysins, called *alpha, beta, gamma* and *delta* lysins are produced by staphylococci. Of these, the alpha lysin (known also as the *alpha* toxin) is the most important in pathogenicity. In cultures, it is produced only under aerobic conditions and its production is enhanced by a high concentration of carbon dioxide. It is a protein which is inactivated at 60°C. Activity is regained paradoxically, if it is further heated to between 80 and

100°C. This is due to the toxin combining with a heat labile inhibitor at 60°C. At higher temperature, the inhibitor is inactivated setting the toxin free. The toxin is denatured above 100°C. The alpha toxin is lytic to rabbit cells, but it is less active with sheep and still less with human red cells. It is also leucocidal, cytotoxic, dermonecrotoxic (producing inflammation and necrosis on intradermal inoculation in rabbit), neurotoxic and lethal (following intravenous inoculation in rabbit). It disrupts lysosomes, damages macrophages and platelets, but monocytes are resistant. It causes injury to the circulatory system, muscle tissue and renal cortex. It has been toxoided and used to stimulate immunity to staphylococci. It is antigenic and is neutralised by the antitoxin.

Beta lysin is haemolytic for sheep, but not for human or rabbit cells. It exhibits a 'hot-cold phenomenon'. Lysis is initiated at 37°C, but is evident only on chilling. It is produced aerobically as well as anaerobically. It has been identified as an enzyme, sphingomyelinase.

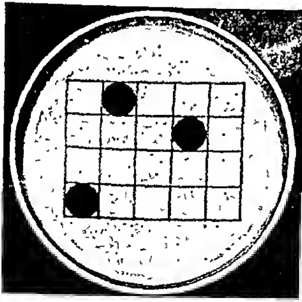

Fig. 22.2 Bacteriophage typing of staphylococci

The gamma lysin, the weakest of the four haemolysins, acts on human, sheep, rabbit and monkey erythrocytes.

The delta lysin is lytic to human and less so to sheep and rabbit erythrocytes.

Alpha and delta lysins are found in strains from human lesions while beta lysin is characteristic of strains of bovine origin.

Leucocidins: This toxin is lethal to the exposed white blood cells of many animals. Its role in pathogenesis is uncertain. Antibodies to leucocidin may play a role in resistance to recurrent staphylococcal infections.

Enterotoxin: This toxin is responsible for the manifestations of staphylococcal food poisoning — nausea, vomiting and diarrhoea occurring within six hours of consuming contaminated food. It is heat stable, resisting boiling for 30 minutes. There are six soluble toxins (A to F), produced almost exclusively by coagulase positive strains of *Staph. aureus*, but not by all such strains, though the majority do so. It is resistant to the actions of gut enzymes. It is produced when *Staph. aureus* grows in carbohydrate and protein foods. The gene for enterotoxin production is probably on the chromosome. The regulation of active toxin production is probably by a plasmid-borne protein. Ingestion of as little as 23 μg of enterotoxin B by humans results in vomiting and diarrhoea. The emetic effect is probably the result of central nervous system stimulation (vomiting centre) after the toxin acts on the neural receptors in the gut.

It is antigenic and is neutralised by the antitoxin. Enterotoxin F is reported to be responsible for the Toxic Shock Sydrome (TSS). It produces diarrhoea in chimpanzees and some monkeys. It has an emetic effect in cats, acting perhaps centrally. By precipitation tests, six antigenic types have been distinguished, A to F, of which, types A and B are the most important. Not all strains produce enterotoxin. Strains producing the toxin usually belong to bacteriophage groups III (6/47) or IV (42D).

Exfoliative toxin: This epidermolytic toxin produced by some strains belonging to phage group II (also of some other phage groups) leads to a variety of exfoliative skin diseases, including generalised exfoliation (Ritter's syndrome), toxic epidermal necrolysis, localised bullous impetigo and generalised scarlatiniform eruption. This clinical picture is called the 'staphylococcal scalded skin syndrome'. This syndrome is usually found in children under four years of age. Two types of the toxin, Exfoliatin A and Exfoliatin B have been described.

In common with streptococcal pyrogenic toxin, *Staph. aureus* produces a toxin which causes a scarlatiniform rash. This toxin may be associated with the scarlatiniform syndrome caused by *Staph. aureus* in persons with Kawasaki disease.

Other toxins: Staphylococci also produce hyaluronidase, fibrinolysin (staphylokinase), nucleases, lipases and proteases, but their pathogenic significance, if any, is not known. Three types of low molecular weight proteins, called pyrogenic exotoxins A, B and C, have been characterised. Type C pyrogenic exotoxin is produced by staphylococcal strains causing the toxic shock syndrome.

Staphylocoagulase: *Staph. aureus* has the property of clotting human or rabbit plasma. This is brought about by the enzyme coagulase, which, along with an activator, the coagulase reacting factor (CRF) present in plasma, converts fibrinogen into fibrin. Clotting does not occur with the plasma of certain species (e.g., guinea pig) because they lack CRF. Seven antigenic types of coagulase have been described. Most human strains produce coagulase A.

When a saline suspension of *Staph. aureus* is mixed on a slide with a drop of plasma, the cocci are clumped. This is due to the precipitation of fibrin on the cell surface by the activity of the 'bound coagulase' or 'the clumping factor'. The free and bound coagulase differ in several respects. Free coagulase is a heat labile enzyme secreted free into the medium, that requires the cooperation of CRF for its action. Bound coagulase is a heat stable constituent of the cell wall and is independent of CRF. Only one antigenic type of bound coagulase has been identified. The two types of coagulase usually go together.

As the criterion for differentiating between *Staph. aureus* and *epidermidis*, coagulase test is a routine laboratory procedure whenever staphylococci are isolated. The test is usually done by two methods, the tube and slide tests.

The tube coagulase test detects free coagulase. Approximately 0.1 ml of an overnight broth culture or an agar culture suspension of the organism is added to approximately 0.5 ml of citrated, oxalated or heparinised human or rabbit plasma (diluted 1 in 5 with saline) in a narrow test tube. Diluted plasma alone in a similar tube serves as the control. The tubes are incubated in a water bath at 37°C for 3–6 hours. If positive, the plasma clots and does not flow when the tube is inverted. On continued incubation, the clot may be lysed by fibrinolysin produced by some strains.

The slide test detects bound coagulase. From an agar culture, the organism is emulsified in two drops of saline placed on a slide. After verifying that there is no spontaneous agglutination, a drop of undiluted human or rabbit plasma is added to one of the emulsions and mixed gently. Prompt clumping of the organism indicates the presence of bound coagulase. Though simple and in general useful, the slide coagulase test should not be used as the sole technique in the diagnostic laboratory. False positive results are caused by citrate utilising bacteria such as enterococci and pseudomonas. In doubtful cases, the tube test should be done for confirmation.

Coagulase promotes the virulence of the organism by inhibiting phagocytosis. It also protects the cells from the bactericidal substances present in tissue fluids. By laying down a fibrin barrier around the cocci, it helps in walling off the lesion.

Pathogenicity: Staphylococci cause the majority of acute pyogenic lesions in man. Staphylococcal

lesions are characteristically localised in contrast to the spreading nature of streptococcal lesions. Staphylococcal diseases may be classified as cutaneous and deep infections, acute toxaemia, including food poisoning, exfoliative diseases and the 'toxic shock syndrome'.

Cutaneous lesions may be of varying severity. These include furuncles, styes, boils, abscesses, carbuncles, impetigo and pemphigus neonatorum. *Staph. aureus* also frequently causes sepsis in wounds and burns. The majority of hospital cross infections are of staphylococcal origin.

The commonest deep infection is acute osteomyelitis, about 90 per cent of which is caused by staphylococci. In the respiratory tract, it causes tonsillitis, pharyngitis, sinusitis and pneumonia. Staphylococcal pneumonia is usually secondary to some other respiratory infection such as influenza. It is usually bronchopneumonic in distribution and may lead to pulmonary abscess. Breast abscess in lactating mothers is an important staphylococcal infection. It may also cause abscesses in other organs, either alone or as part of pyaemia. Staphylococcal septicaemia is a rare, but serious disease. A haematogenous spread may also lead to meningitis, endocarditis and renal abscesses. Tropical pyomyositis is a condition observed in young adults in many parts of the tropics, characterised by multiple staphylococcal abscesses in voluntary muscles.

Staphylococcal food poisoning results when food contaminated with enterotoxin-producing staphylococci is consumed. The types of food usually responsible are meat, fish, milk and milk products. Sufficient time should elapse between staphylococcal contamination of the food and its consumption so that the organism can grow and produce enough enterotoxin. Diarrhoea and vomiting set in within about six hours of taking the contaminated food. Some cases of post-antibiotic diarrhoea are caused by enterotoxin producing strains of staphylococci.

Stripping of the superficial layers of the skin from underlying tissues by the action of the exfoliative (epidermolytic) toxin is the pathogenic mechanism in the various exfoliative syndromes caused by staphylococci (bullous impetigo, pemphigus neonatorum, Ritter's disease, toxic epidermal necrolysis).

Toxic shock syndrome (TSS) is a multisystem illness characterised by acute onset of high fever, hypotension, vomiting, diarrhoea and a scarlatiniform rash with subsequent desquamation. Severe cases progress to acute renal failure, disseminated intravascular coagulation, peripheral gangrene and death. Though TSS became widely known in association with the use of tampons by menstruating women in the USA, it occurs in other situations also. TSS is caused by staphylococci producing enterotoxin F, usually belonging to phage Group I.

Epidemiology: Staphylococci are ubiquitous in nature, but the commonest sources of infection are human patients and carriers, animals and inanimate objects being less important. Patients with superficial infections and respiratory infections disseminate large numbers of staphylococci into the environment. About 10–30 per cent of healthy persons carry staphylococci in the nose and about 10 per cent in the perineum and also on the hair. Staphylococcal carriage starts early in life, colonisation of the umbilical stump being very common in babies born in hospitals. Some carriers, called 'shedders', disseminate very large numbers of cocci for prolonged periods. The cocci shed by patients and carriers contaminate fomites such as handkerchiefs, bed linen and blankets, and may persist on them for days or weeks. Staphylococci may also come from infected domestic animals such as cows.

Staphylococcal disease may follow endogenous or exogenous infection. The modes of transmission may be by contact, direct or through fomites, by dust or by airborne droplets.

Hospital infections by staphylococci deserve special attention because of their frequency and because they are caused by strains resistant to various antibiotics. Staphylococci are the commonest cause of post-operative wound infections and other hospital cross infections. Most of these are due to certain strains of staphylococci that are

present in the hospital environment, the so-called 'hospital strains'. They belong to a limited number of phage types and are commonly resistant to penicillin and other antibiotics routinely used in hospitals. Some of them, the 'epidemic strains' cause epidemics of hospital cross infections. The first of these to be recognised was phage type 80/81, which accounted for most of staphylococcal infections in hospitals throughout the world. From 1957, they have been replaced by strains of phage group III (type 83A).

Measures for the control of staphylococcal infection in hospitals include, 1) isolation of patients with open staphylococcal lesions, 2) detection of staphylococcal lesions among surgeons, nurses and other hospital staff and keeping them away from work till the lesions are healed, and 3), strict aseptic techniques in theatres. If an outbreak of staphylococcal sepsis occurs, a search may be made for carriers among the hospital staff. Those detected should be treated with local applications of neomycin and chlorhexidine. In some institutions in America, eradication of the virulent resident strain has been attempted by the deliberate dissemination of a strain of low virulence. The latter may oust the former by interference. Antimicrobial prophylaxis by topical applications of antiseptics such as hexachlorophene also has been found useful.

Laboratory diagnosis: The specimens to be collected depend on the type of lesion (e.g., pus from suppurative lesions, sputum from respiratory infections). In cases of food poisoning, faeces and remains of suspected food should be collected. For detection of carriers, the usual specimen is the nasal swab; swabs from the perineum, pieces of hair and umbilical stump may be necessary in special situations.

Direct microscopy with Gram stained smears is useful with pus, where cocci in clusters may be seen. This is of no value with specimens like sputum where mixed bacterial flora are normally present.

Diagnosis may readily be made by culture. The specimens are plated on blood agar. Staphylococcal colonies appear after overnight incubation. Specimens where staphylococci are expected to be scanty or outnumbered by other bacteria (e.g. swabs from carriers, faeces from food poisoning) are inoculated on selective media like Ludlam's or salt-milk-agar or Robertson's cooked meat medium containing 10 per cent sodium chloride. Smears are examined from the cultures and the coagulase test done when staphylococci are isolated. Antibiotic sensitivity tests should be performed as a guide to treatment. This is important as staphylococci develop resistance to drugs readily. Bacteriophage typing may be done if the information is desired for epidemiological purposes.

Serological tests may sometimes be of help in the diagnosis of hidden deep infections. Antistaphylolysin (anti-alphalysin) titre of more than two units per ml, especially when the titre is rising, may be of value in the diagnosis of deep-seated infections such as bone abscess.

Treatment: As drug resistance is so common among staphylococci, the appropriate antibiotic should be chosen by antibiotic sensitivity tests. Benzyl penicillin is the most effective antibiotic, if the strain is sensitive. Methicillin and cloxacillin are effective against penicillinase producing strains. For mild superficial lesions, systemic antibiotics may not be necessary. Topical applications of drugs not used systemically, such as bacitracin, may be sufficient.

Some strains show the phenomenon of drug tolerance. These strains will be found as susceptible in the disc sensitivity test, but their minimum bactericidal concentration will be very much higher than their minimum inhibitory concentration. They are not killed by antibiotics in the usual doses and persist, leading to failure in eradicating the infection.

The treatment of carriers is by local applications of suitable antibiotics and antiseptics.

For the treatment of some chronic infections such as recurrent furuncles, resistant to the usual therapeutic measures, autovaccines have been tried, sometimes with considerable success. Antisera are of no value in treatment.

Staphylococcus Intermedius

Some coagulase positive strains, with characters intermediate between *Staph. aureus* and *epidermidis* have been designated *Staph. intermedius*. They are mainly of animal origin.

Coagulase negative staphylococci

The classification of coagulase negative staphylococci is still unsettled. They are generally grouped into two species, *Staph. epidermidis* and *Staph. saprophyticus*, each with many biotypes.

Staphylococcus epidermidis

These are coagulase negative staphylococci which are part of the skin flora. They are usually nonpathogenic, but are common contaminants being derived from the skin during collection of specimens. They may act as opportunist pathogens, causing minor infections such as stitch abscesses, and in persons with defective resistance, even serious illness such as septicaemia. In persons with structural abnormalities of the urinary tract, they may produce cystitis. They have been reported to cause subacute endocarditis. They have been found commonly to colonise some surgical prostheses such as the Spitz-Holter valve. It is often difficult to decide their clinical significance when isolated from specimens. Their aetiological role may have to be proved by repeated isolation.

Staphylococcus saprophyticus

This coagulase negative staphylococcus has become clinically important as a common cause of acute urinary tract infection, particularly in young women. The infection is usually self-limited but may occasionally lead to renal involvement. It may also cause urethritis. It differs from *Staph. epidermidis* in being resistant to novobiocin.

Micrococci

These are Gram positive, cluster-forming cocci which differ from staphylococci in attacking sugars oxidatively. They may occur in irregular clusters, in groups of four (tetrads as in *Gaffkya tetragena*) or of eight (as in *Sarcina lutea*). The cocci are usually of irregular size and often larger than staphylococci. Their staining is also often not uniform. They are saprophytes and commensals that may rarely cause opportunistic infections.

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- established pathogenicity by inoculation ·

23 Streptococcus

Streptococci are Gram positive cocci arranged in chains. They are important human pathogens, causing pyogenic infections, with a characteristic tendency to spread, as opposed to staphylococcal lesions, which are typically localised. They are also responsible for nonsuppurative lesions, acute rheumatic fever and glomerulonephritis, which occur as sequelae to streptococcal infection.

Cocci in chains were first seen in erysipelas and wound infections by Billroth (1874), who called them streptococci (*streptos*, meaning twisted or coiled). Ogston (1881) isolated them from acute abscesses, distinguished them from staphylococci and established their pathogenicity by animal inoculation. Rosenbach (1884) isolated the cocci

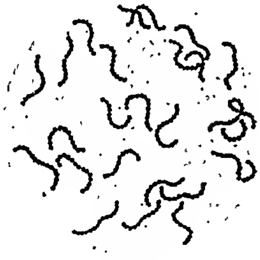

Fig. 23.1 Streptococci

from human suppurative lesions and gave the name *Streptococcus pyogenes*.

Classification of streptococci

Several systems of classification have been employed, but in medical bacteriology, the following method is useful (Fig. 23.2).

Streptococci are first divided into obligate anaerobes and facultative anaerobes. The former are designated peptostreptococci and are considered in a later chapter. The aerobic and facultative anaerobic streptococci are classified, based on their haemolytic properties. Haemolysis was first proposed as a criterion for classification by Schottmuller (1903), but it was established on a firm basis by Brown (1919). Employing meat infusion peptone agar with 5% horse blood, Brown recognised three types of reactions.

1. *Alpha* (α) haemolytic streptococci produce a greenish discolouration with partial haemolysis around the colonies. The zone of lysis is small (1 or 2 mm wide), with indefinite margins and unlysed erythrocytes can be made out microscopically within this zone.

2. *Beta* (β) haemolytic streptococci produce a sharply defined, clear, colourless zone of haemolysis, 2 or 4 mm wide, within which red cells are completely lysed.

3. *Gamma* (γ) or nonhaemolytic streptococci produce no change in the medium. These are sometimes called *indifferent streptococci*.

Most of the pathogenic streptococci fall into the beta group and are called the 'haemolytic

Streptococci

O_2 requirement

Aerobes and facultative anaerobes

Obligate anaerobes
Peptostreptococci

Haemolysis — meat infusion peptone agar + 5% horse blood.

1. Alpha
(The viridans group)
Classified into species by physiological and biochemical properties

greenish zone of discoloration
1–2 mm wide
indefinite margins
ē nig haemolysed
surfaces ..... seen

2. Beta
(The haemolytic streptococci)
— clear colourless zone of discoloration

Serological Grouping (Polysaccharide C)

19 Lancefield Groups (ABCDEFGHKL MNOPQRSTU)V

A – V (19) (⊗ I; J)

3. Gamma
(The enterococcus group) faecal str.
Classified into species by physiological and biochemical properties
= indifferent streptococci

human infection — Group A · *Streptococcus pyogenes*

Serological typing (M Protein)

Griffith types (1,2,3, etc.)

Fig 23.2 Classification of streptococci

streptococci'. The alpha streptococci are generally commensals in the throat, though they may at times produce opportunistic infections. Because of the distinctive green colour they produce, they are called the 'viridans group' (*Str. viridans*). The gamma streptococci include the faecal streptococci (*Str. faecalis*, enterococcus) and related species. They are called the 'enterococcus group'.

The haemolytic streptococci were classified by Lancefield (1933) serologically into groups based on the nature of a carbohydrate (C) antigen on the cell wall. These are known as Lancefield groups, 19 of which have been identified so far and named A–V (without I and J). The great majority of haemolytic streptococci that produce human infections belong to group A. Haemolytic streptococci of group A are known as *Str. pyogenes*. These may be further subdivided into types based on the protein (M, T and R) antigens present on the cell surface (Griffith typing). About eighty types of *Str. pyogenes* have been recognised so far.

Streptococcus pyogenes

Morphology: The individual cocci are spherical or oval, 0.5–1.0 μ in diameter. Since variations result from cultural conditions, e.g., when grown anaerobically, they are somewhat smaller. They are arranged in chains, the length of which varies within wide limits and is influenced by the nature

Media for strep. since staph. are more resistant to crystal violet unlike strep.

of the culture medium, chains being longer in liquid than in solid media. Chain formation is due to the cocci dividing in one plane only and the daughter cells failing to separate completely. There is often an appearance of pairing within the chains. Significance was once attached to the length of the chains and streptococci had been classified accordingly (*Str. longus* and *brevis*), but this has no relevance to virulence or other properties. In fact, some nonpathogenic streptococci form the longest chains, e.g., *Str. salivarius*.

Streptococci are nonmotile and nonsporing. Some strains of *Str. pyogenes* and some group C strains have capsules composed of hyaluronic acid while polysaccharide capsules are encountered in members of groups B and D. These capsules are best seen in very young cultures.

L forms. Protoplasts may be induced by penicillin or phage associated lysin and may be propagated on hypertonic media to produce L forms or colonies. Removal of penicillin usually allows reversion to parent strains. However, there may be stable L form strains that are no longer capable of reversion. The role of L forms in disease states or in the persistence of streptococci in tissues is unknown.

Cultural characteristics. It is an aerobe and facultative anaerobe, growing best at a temperature of 37°C (range 22°–42°C). It is exacting in nutritive requirements, growth occurring only in media containing fermentable carbohydrates or enriched with blood or serum. On blood agar, after incubation for 24 hours, the colonies are small (0.5–1.0 mm) circular, semitransparent, low convex discs with an area of clear haemolysis around them. Growth and haemolysis are promoted by 10 per cent CO_2. Virulent strains, on fresh isolation from lesions, produce a 'matt' (finely granular) colony, while avirulent strains form 'glossy' colonies. Strains with well marked capsules produce 'mucoid' colonies, corresponding in virulence to the matt type. Very rarely, nonhaemolytic group A streptococci are encountered, which are typical of *Str. pyogenes* in other respects.

In liquid media, such as glucose or serum broth, growth occurs as a granular turbidity with a powdery deposit. No pellicle is formed.

Biochemical reactions: Streptococci ferment several sugars producing acid but no gas. Fermentation of sugars (sorbitol, trehalose, lactose, maltose, dextrin, mannitol) has been made the basis of a physiological classification of the genus, but this has been replaced by serological grouping in the case of haemolytic streptococci.

Streptococci are catalase negative. They are not soluble in 10 per cent bile, unlike pneumococci.

Resistance: *Str. pyogenes* is a delicate organism, easily destroyed by heat (54° for 30 minutes). It dies in a few days in cultures, unless stored at a low temperature (4°), preferably in Robertson's cooked meat medium. It can, however, survive in dust for several weeks, if protected from sunlight. It is rapidly inactivated by antiseptics. It is more resistant to crystal violet than many bacteria, including *Staph. aureus*, and hence this dye is used for preparation of selective media. It is susceptible to sulphonamides and many antibiotics, but unlike *Staph. aureus*, does not develop resistance to drugs. Sensitivity to bacitracin is employed as a convenient method for differentiating *Str. pyogenes* from other haemolytic streptococci.

no resistance

Antigenic structure: Fig. 23.3 illustrates the disposition of various antigens in *Str. pyogenes*. The capsule when present inhibits phagocytosis. It is not antigenic in man.

The cell wall is composed of an outer layer of fimbria containing protein and lipoteichoic acid, a middle layer of group specific carbohydrate and an inner layer of peptidoglycan.

The peptidoglycan (mucoprotein) is responsible for cell wall rigidity. It has also some biological properties such as pyogenic and thrombolytic activity.

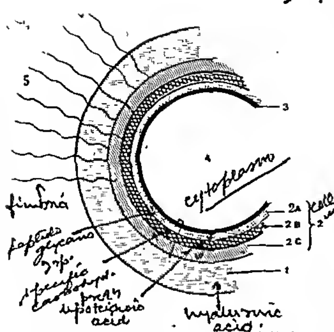

Fig. 21.3 Antigenic structure of *Str. pyogenes*. 1 Hyaluronic acid capsule, 2 cell wall comprising 2A peptidoglycan, 2B group specific carbohydrate and 2C protein lipoteichoic acid fimbria 3 cytoplasmic membrane 4 cytoplasm 5 pili covered with lipoteichoic acid

Serological grouping of streptococci depends on the C carbohydrate. *Str. pyogenes* belongs to group A. As this antigen is an integral part of the cell wall, it has to be extracted for grouping by a precipitation test with group antisera. For the test, streptococci are grown in Todd-Hewitt broth and extracted with HCl (Lancefield's acid extraction method), or formamide (Fuller's method) or by an enzyme produced by *Streptomyces albus* (Maxted's method) or by autoclaving (Rantz and Randall's method). The extract and the specific antisera are allowed to react in capillary tubes. Precipitation occurs within five minutes at the interface between the extract and the homologous antiserum. Grouping may also be done by agar gel precipitation.

Several protein antigens have been identified in the outer part of the cell wall. *Str. pyogenes* can be typed, based on the surface proteins M, T and R. The M protein is the most important of these. It acts as a virulence factor by inhibiting phagocytosis. It is antigenic. The antibody to the M protein promotes phagocytosis of the coccus and is therefore protective. The M protein is acid and heat labile, susceptible to tryptic digestion and soluble in alcohol. It can be extracted by the Lancefield acid extraction method and typing is done with type specific sera. About 80 M protein types have been recognised.

The T protein is an acid labile, trypsin resistant antigen present in many serotypes of *Str. pyogenes*. It may be type specific, but many different M types possess the same T antigen. It is usually demonstrated by agglutination with specific sera. Some types of *Str. pyogenes* (types 2, 3, 28 and 48) and some strains of groups B, C and G contain a third antigen, the R protein. The T and R proteins have no relation to virulence. A non-typespecific protein has been identified, associated with the M protein. This is known as M associated protein (MAP). Many types of *Str. pyogenes* have a serum opacity factor (SOF) also. Culture supernates of these strains produce opacity when applied to agar gel containing horse or swine serum.

Hair-like pili project through the capsule of group A streptococci. The pili consist partly of M protein and are covered with lipoteichoic acid which is important in the attachment of streptococci to epithelial cells.

Various structural components of *Str. pyogenes* exhibit antigenic cross reaction with different tissues of the human body. Antigenic relationships have been demonstrated between capsular hyaluronic acid and human synovial fluid, cell wall protein and myocardium, group A carbohydrate and cardiac valves, cytoplasmic membrane antigens and vascular intima, and peptidoglycans and skin antigens. It has been postulated that these antigenic cross reactions may account for some of the manifestations of rheumatic fever and other streptococcal diseases, the tissue damage being of an immunological nature.

Toxins and other virulence factors

Str. pyogenes forms several exotoxins and enzymes which contribute to its virulence. Be-

Besides these, the M protein also acts as a virulence factor by inhibiting phagocytosis. The C polysaccharide has been shown to have a toxic effect on connective tissues in experimental animals.

Haemolysins: Streptococci produce two haemolysins, streptolysin 'O' and 'S'. Streptolysin O is so called because it is oxygen labile. It is inactive in the oxidised form, but may be reactivated by treatment with mild reducing agents. On blood agar, streptolysin O activity is seen only in pour plates and not in surface cultures. It may be obtained in the active state by growing streptococci in broth containing reducing agents such as sodium hydrosulphite. It is also heat labile. It appears to be important in contributing to virulence. It is lethal on intravenous injection into animals and has a specific cardiotoxic activity. It has leucotoxic activity also. In its biological action, streptolysin O resembles the oxygen labile haemolysin of *Cl. perfringens*, *Cl. tetani* and the pneumococcus.

Streptolysin O is antigenic and antistreptolysin regularly appears in sera following streptococcal infection. Estimation of this antibody (ASO titre) is a standard serological procedure for the retrospective diagnosis of infection with *Str. pyogenes*. The lysin is inhibited by cholesterol, but not by normal sera. Following certain chemical treatments or bacterial contamination, sera may develop inhibitory activity due to some changes in the lipoproteins. Such sera are unfit for ASO test. An ASO titre in excess of 160–200 units is considered abnormally high and suggests either recent infection with streptococci or due to an exaggerated immune response to an earlier exposure in a hypersensitive person. Streptolysin S and O are produced by groups A, C and G.

Streptolysin S is an oxygen stable haemolysin and so is responsible for the haemolysis seen around streptococcal colonies on the surface of blood agar plates. It is called streptolysin S since it is soluble in serum. It can also be obtained by growing the cocci with ribonucleic acid. Streptolysin S is largely bound to the cell and its release requires the participation of some carrier molecule (serum albumin or RNA). It is a protein, but is not antigenic. Convalescent sera do not neutralise streptolysin S activity. It is inhibited nonspecifically by serum lipoproteins. It has been shown experimentally to be nephrotoxic, but its significance in pathogenesis is not understood.

Erythrogenic (Dick, scarlatinal) toxin: It was long believed that the scarlatiniform rash of scarlet fever was a primary reaction to the toxin. It is now generally accepted that the erythrogenic activity is an expression of the enhancement of hypersensitivity to other streptococcal products and, that the primary toxicity of these substances is manifest as pyrogenicity through hypothalamic stimulation; enhanced susceptibility to endotoxic shock, lymphocyte mitogenesis and suppression of antibody response by alteration of the activity of lymphocytes. Hence they are more appropriately termed streptococcal pyrogenic exotoxins. Three distinct immunological types, A, B and C, have been described, though they show some immunological cross reaction. They stimulate neutralising antibodies. Only group A streptococci are known to form pyrogenic exotoxins but not all of its strains do so. Toxin production is apparently due to lysogenic conversion by bacteriophage.

The toxin is thermostable and antigenic. It is neutralised by antibodies found in the convalescent sera. This property has been used for developing susceptibility and diagnostic tests for scarlet fever (Dick test and Schultz-Charlton reaction). These tests are now only of historical importance as scarlet fever is no longer a common or serious disease.

Streptokinase (Fibrinolysin): This toxin promotes the lysis of human fibrin clots by activating a plasma precursor (plasminogen). It is an antigenic protein and neutralising antibodies appear in convalescent sera. These may be estimated and provide retrospective evidence of streptococcal infection. Fibrinolysin appears to play a biological role in streptococcal infections by breaking down the fibrin barrier around the lesions and facilitating the spread of infection. Streptokinase

has been given intravenously for treatment of pulmonary emboli and venous thrombosis.

ABCD

Deoxyribonucleases (Streptodornase): These cause depolymerisation of DNA. Pyogenic exudates contain large amounts of DNA, derived from the nuclei of necrotic cells. Streptodornase helps to liquefy the thick pus and may be responsible for the thin serous character of streptococcal exudates. This property has been applied therapeutically in liquefying localised collections of thick exudates, as in empyema. A preparation containing streptokinase and streptodornase is available for this purpose. Four antigenically distinct streptodornases, A, B, C and D, have been recognised, of which, type B is the most antigenic in man. Streptodornase types B and D also possess ribonuclease activity. Streptodornase B is antigenic and demonstration of the antibody is useful in the retrospective diagnosis, particularly of skin infections, where ASO titre may be low.

Diphosphopyridinenucleotidase (DPN-ase): This acts on the co-enzyme DPN and liberates nicotinamide from the molecule. It is antigenic and is specifically neutralised by the antibody in convalescent sera. The biological significance of DPN-ase is not known, though it is believed to be leucotoxic.

Hyaluronidase: This enzyme breaks down the hyaluronic acid of the tissues. This might favour the spread of infection along intercellular spaces. Streptococci possess a hyaluronic acid capsule and also elaborate a hyaluronidase — a seemingly self-destructive process. But it is found that strains that form hyaluronidase in large quantities (M types 4 and 22) are noncapsulated. There is some evidence that strains that do not form detectable amounts of hyaluronidase in cultures may, nevertheless, do so in tissues. The enzyme is antigenic and specific antibodies appear in convalescent sera.

Proteinase: This is another instance of an apparently self-destructive enzyme, since it is capable of breaking down the M protein as well as streptokinase and hyaluronidase. The enzyme is, however, produced only under special conditions, such as an acid pH (5.5–6.5). Such conditions may be produced by tissue destruction, as in abscessess. The biological significance of this enzyme is not understood.

NAD-ase

Many strains also produce nicotinamide adenine dinucleotidase (NAD-ase), phosphatase, esterases, amylase, N acetyl glucosaminidase, neuraminidase and a cardiohepatic toxin possibly distinct from erythrogenic toxin. It is not known whether, and to what extent, these contribute to pathogenesis.

Pathogenicity

resp. infns
skin infection
genital
suppurative
non-suppurative

Str. pyogenes produces pyogenic infections with a tendency to spread locally, along lymphatics and through the bloodstream.

1. Respiratory infections: The primary site of invasion of the human body by *Str. pyogenes* is the throat. Sore throat is the commonest of streptococcal diseases. It may be localised as tonsillitis or may involve the pharynx more diffusely (pharyngitis). Virulent group A streptococci adhere to the pharyngeal epithelium by means of lipoteichoid acid covering surface pili. The glycoprotein fibronectin on epithelial cells probably serves as lipoteichoid acid ligand. Tonsillitis is commoner in older children and adults than in younger children, who commonly develop diffuse pharyngitis. Localisation is believed to be favoured by hypersensitivity due to prior contact. In pharyngeal infection in children, abdominal pain is occasionally complained of and may be confused with appendicitis. Pharyngitis is mainly caused by group A, though sporadic cases and epidemics have been reported due to groups C and G. Rheumatic fever may occur in upto three per cent of individuals during epidemic pharyngitis.

Scarlet fever is a special variety of streptococcal sore throat where the infection is caused by a strain producing the erythrogenic toxin. This accounts for the characteristic erythematous

rash. Scarlet fever, once a very serious disease of colder countries, causing epidemics in institutions such as boarding schools is now but rarely seen. This is uncommon in the tropics and does not occur in India.

From the throat, streptococci may spread to the surrounding tissues, leading to suppurative complications, such as otitis media, mastoiditis, quinsy, Ludwig's angina and suppurative adenitis. It may rarely lead to meningitis. Streptococcal pneumonia seldom follows throat infection but may occur as a complication of influenza or other respiratory viral diseases.

2. *Skin infections*: *Str. pyogenes* causes a variety of suppurative infections of the skin, including infection of wounds or burns, with a predilection to produce lymphangitis and cellulitis. Infection of minor abrasions may at times lead to fatal septicaemia.

The two typical streptococcal infections of the skin are erysipelas and impetigo. The former is a diffuse infection involving the superficial lymphatics. The affected skin, which is red, swollen and indurated, is sharply demarcated from the surrounding healthy area. While erysipelas is commoner in older patients, impetigo is found mainly in young children. Impetigo is caused by *Str. pyogenes* belonging to a limited number of serotypes only. Impetigo and streptococcal infection of scabies lesions are the main causes leading to acute glomerulonephritis in children in the tropics.

In pyoderma, antibody response to streptolysin 'O' is not high and ASO estimation does not have much clinical significance as in pharyngeal infections. Antibody to DNA-se B and hyaluronidase are more useful in retrospective diagnosis of pyoderma antecendent to acute glomerulonephritis.

3. *Genital infections*: Both aerobic and anaerobic streptococci are normal inhabitants of the female genitalia. *Str. pyogenes* is an important cause of puerperal sepsis, but here, the infection is usually introduced from without. Anaerobic streptococcal puerperal infection is more often endogenous. Puerperal fever caused by *Str. pyogenes* used to take a heavy toll of life before aseptic practices were introduced for delivery.

4. *Other suppurative infections*: *Str. pyogenes* may cause abscesses in internal organs such as the brain, lung, liver and kidney, and may also cause septicaemia and pyaemia.

5. *Nonsuppurative complications*: *Str. pyogenes* infections lead to two important nonsuppurative

TABLE 23.1

Comparison of rheumatic fever and glomerolonephritis

	Acute rheumatic fever	*Acute glomerulonephritis*
Site of infection	Throat	Throat or skin
Prior sensitisation	Essential	Not necessary
Serotype of *Str. Pyogenes*	Any	Pyoderma types 2, 49, 57, 59-61 and pharyngitis strains 1 and 12 (lead on to acute glomerulonephritis.)
Immune response	Marked	Moderate
Complement level	Unaffected	Lowered
Hereditary tendency	Present	Not known
Repeated attacks	Common	Absent
Penicillin prophylaxis	Essential	Not indicated
Course	Progressive or static	Spontaneous resolution
Prognosis	Variable	Good

sequelae — acute rheumatic fever and acute glomerulonephritis. These complications ensue one to three weeks after the acute infection so that the organism is no longer detectable when sequelae set in. They differ in a number of respects as to their natural history (Table 23.1).

The pathogenesis of these complications is not clearly understood. The essential lesion in rheumatic fever is carditis, including connective tissue degeneration of the heart valves and inflammatory myocardial lesions characterised by Aschoff nodules. The lesions are believed to be the result of hypersensitivity to some streptococcal component, produced by repeated infections. It has also been suggested that there may be an element of autoimmunity involved and antigenic cross reactions have been demonstrated between streptococci and heart tissues. Lesions resembling rheumatic fever have been produced experimentally in rabbits by repeated infection with *Str. pyogenes* and in mice by injection of sonic lysates of the cocci.

While rheumatic fever may follow infection with any serotype of *Str. pyogenes*, nephritis is caused by only a few 'nephritogenic' types. In the tropics, skin infections are perhaps more important in this respect than throat infections. The nephritis is usually a self-limited episode that resolves without any permanent damage. The pathogenesis may be due to antigenic cross reactions between a glomerular antigen and some component of the nephritogenic streptococci, or more often it may be an immune complex disease. This condition has been produced in monkeys and rabbits by repeated infection with type 12 *Str. pyogenes* or injection of bacterial products, and in mice, with soluble streptococcal products.

Epidemiology: The major source of *Str. pyogenes* is the human upper respiratory tract — throat, nasopharynx or nose — of patients and carriers. Symptomless infection is common and helps to maintain the organism in the community. Transmission of infection is either by direct contact or through contaminated dust or fomites. An important nonhuman source of infection is bovine mastitis, the organism being transmitted to man through milk.

Streptococcal infections of the respiratory tract are more frequent in children 5–8 years of age than in young children below two years or in adults. They are commoner in winter in the temperate countries. No seasonal distribution has been identified in the tropics. Crowding is an important factor in the transmission of infection. Outbreaks of infection may occur in closed communities such as boarding schools or army camps.

Immunity is type specific and appears to be associated with antibody to the M protein. Reinfections occur because of the multiplicity of serotypes.

Laboratory diagnosis: In acute infections, diagnosis is established by culture, while in the nonsuppurative complications, diagnosis is based on the demonstration of antibodies.

Presumptive information may be obtained by an examination of Gram stained films from pus and CSF. The presence of Gram positive cocci in chains is indicative of streptococcal infection. But smears are of no value in infections of the throat or genitalia, where streptococci may form part of the resident flora.

For cultures, swabs should be collected under vision from the affected site and either plated immediately or sent to the laboratory in Pike's medium (blood agar containing 1 in 1,000,000 crystal violet and 1 in 16,000 sodium azide). The specimen is plated on blood agar and incubated at 37°C anaerobically or under 5–10% CO_2, as haemolysis develops better under these conditions. Sheep blood agar is recommended for primary isolation because it is inhibitory for *Haemophilus haemolyticus*, colonies of which may be confused with those of haemolytic streptococci. Haemolytic streptococci are grouped by the Lancefield technique. The fluorescent antibody technique has been employed for the rapid identification of group A streptococci. A convenient method for the identification of *Str. pyogenes* is based on Maxted's observation that they are more sensitive to bacitracin than other

streptococci. A filter paper disc dipped in a solution of bacitracin (1 unit/ml) is applied on the surface of an inoculated blood agar. After incubation, wide zone of inhibition is seen with *Str. pyogenes*, but not with other streptococci.

Typing of *Str. pyogenes* is required only for epidemiological purposes. If required, this may be done by precipitation or agglutination. A phage typing scheme has also been proposed.

In rheumatic fever and glomerulonephritis, a retrospective diagnosis of streptococcal infection may be established by demonstrating high levels of antibody to streptococcal toxins. The usual test done is antistreptolysin O titration. ASO titres higher than 160 or 200 are indicative of prior streptococcal infection. High levels are usually found in acute rheumatic fever, but in glomerulonephritis, titres are often low. Antideoxyribonuclease B (ADN-ase B) estimation is also commonly employed. Titres higher than 300 or 350 are taken as significant. ADN-ase B and antihyaluronidase tests are very useful for the retrospective diagnosis of streptococcal pyoderma, for which ASO is of much less value.

The streptozyme test, a passive slide haemagglutination test using erythrocytes sensitised with a crude preparation of extracellular antigens of streptococci, is a convenient, sensitive and specific screening test. It becomes positive after nearly all types of streptococcal infection, whether of the throat or the skin.

Prophylaxis: The indication for prophylaxis in streptococcal infection is only in the prevention of rheumatic fever. This is achieved by a long-term administration of penicillin in children who have developed early signs of rheumatic fever. This prevents streptococcal reinfection and further damage to the heart. Antibiotic prophylaxis is not useful for glomerulonephritis as this complication follows a single streptococcal infection and reinfections do not occur.

Treatment: All beta haemolytic group A streptococci are sensitive to penicillin G, and most are sensitive to erythromycin. In patients allergic to penicillin, erytbromycin or cephalexin may be used. Strains resistant to erythromycin have been reported. Tetracyclines and sulphonamides are not recommended. Antimicrobial drugs have no effect on established glomerulonephritis and rheumatic fever.

Other haemolytic streptococci

Besides *Str. pyogenes*, haemolytic streptococci belonging to groups B, C, D, F, G, H, K and O may also cause human infections.

Data from Streptococcal Reference Laboratories in India (Lady Hardinge Medical College, New Delhi; Christian Medical College, Vellore) show that, while approximately 45 per cent of haemolytic streptococcal isolates tested belong to group A, 10–15 per cent belong to groups B and C each, about 25 per cent to group G and 5 per cent to group F.

Group B: These are important pathogens of cattle, producing bovine mastitis *(Str. agalactiae)*. In recent years, Group B streptococcus has assumed great clinical importance as the single most common cause of neonatal meningitis. Infection in the newborn is classified as the *early onset* type, occurring within a week of birth, and the *late onset* type disease developing between the 2nd and 4th weeks of life. The more common early onset type presents as septicaemia, meningitis or pneumonia, and is often fatal. Infection is acquired from the maternal vagina during birth. In the late onset type, infection is more often obtained from the environment. The late onset type though not as severe as the early onset ones, has a high incidence of residual effects, often of a neurological nature. Group B streptococci may also cause adult infections, including puerperal sepsis.

Their ability to hydrolyse hippurate acts as a presumptive identification method. They may be identified by the CAMP reaction (Christie, Atkins and Munch-Peterson), which is the production of lysis when staphylococcal beta haemolysin is poured on a plate on which streptococci are

grown. Occasional strains are bacitracin sensitive. Five serological types have been identified.

Group C: Streptococci of this group are predominantly animal pathogens and may be divided into four species biochemically. They are frequently present in the human throat and may cause pharyngitis and other infections.

Group F: These grow poorly on blood agar unless incubated under CO_2. They have been called the 'minute streptococci'. They are sometimes found in suppurative lesions.

Group G: These are commensals in the throat of man, monkey or dogs. They may occasionally cause tonsillitis, endocarditis and urinary infections in man.

Groups H and *K* sometimes cause infective endocarditis.

Group O is isolated mainly from the healthy human throat. It is suspected to cause acute tonsillitis and endocarditis.

The viridans group (*Str. viridans*)

The group consists of streptococci that produce alpha haemolysis on blood agar. Based on biochemical reactions, they have been classified into five species (*Str. salivarius, mutans, sanguis, mitior* and *milleri*). They are constantly present in the human mouth and throat. They are normally nonpathogenic, but in persons with predisposing factors, such as valvular disease of the heart, they produce subacute bacterial endocarditis. Tooth extraction in such persons is dangerous, because of the bacteraemia that follows, and should be done under antibiotic cover.

Diagnosis of subacute bacterial endocarditis is established by repeated blood cultures. These streptococci are generally susceptible to penicilin, though some strains may be resistant. A determination of antibiotic sensitivity is therefore necessary in planning proper treatment.

Dental caries has been attributed to the activity of *Str. mutans*, which breaks down sucrose, producing acid and a tough adhesive dextran. The acid damages dentine and the dextrans bind together food debris, epithelial cells, mucus and bacteria to form dental plaques, which lead to caries. Experimental caries in monkeys have been prevented by *Str. mutans* vaccine.

Group-D streptococci

Streptococci belonging to group D are classified as the faecal streptococci (enterococci) comprising the species *Str. faecalis*, *Str. faecium* and *Str. durans*, and the nonfaecal species *Str. bovis* and *Str. equinus*. They may or may not be haemolytic.

Enterococci possess some distinctive properties such as the ability to grow in the presence of 40 per cent bile, 6.5 per cent sodium chloride, at pH 9.6, at 45°C and in 0.1 per cent methylene blue milk. On MacConkey's medium they produce tiny, deep pink colonies. They are relatively heat resistant, surviving 60°C for 30 minutes. Enterococci typically appear as pairs of oval cocci, the cocci in a pair arranged at an angle to each other (Fig. 23.4).

The identification of the species is made on biochemical grounds. *Str. faecalis* is the enterococcus most often isolated from human sources. *Str. faecalis* can be identified by its

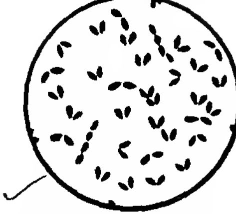

Fig. 23.4 Enterococcus. Oval cells arranged in pairs at an angle, or in short chains

ability to ferment mannitol, sucrose, sorbitol and aesculin, and to grow on tellurite blood agar producing black colonies.

Enterococci are present in the intestine, genital tract and saliva. They are frequently isolated from cases of urinary tract infection and wound infection. They may also cause subacute bacterial endocarditis, infection of the biliary tract, septicaemia, peritonitis and intraabdominal abscess complicating diverticulitis and peritonitis. Strains resistant to penicillin and other antibiotics occur frequently so that it is essential to perform antibiotic sensitivity for proper therapy.

Nonfaecal species of group D (*Str. bovis*, *Str. equinus*) are generally susceptible to penicillin and are inhibited by 6.5 per cent sodium chloride or bile. They may cause urinary infection or endocarditis rarely.

Streptococcus MG

This is an α streptococcus belonging to group F. It may be present in the sputum of normal individuals and has been isolated from the lungs and throat washings of cases of primary atypical pneumonia. Patients with primary atypical pneumonia frequently have in their sera agglutinins to streptococcus MG.

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24 Pneumococcus

(Diplococcus pneumoniae: Str. pneumoniae)

Pneumococci are Gram positive, lanceolate diplococci which resemble the viridans streptococci, with which they were classified formerly. The pneumococcus has been reclassified as *Str. pneumoniae* because of its genetic relatedness to streptococcus. They differ from streptococci chiefly in their morphology, bile solubility, optochin sensitivity and possession of a specific polysaccharide capsule. Pneumococci are normal inhabitants of the upper respiratory tract of humans. They are the most prevalent single bacterial agent in pneumonia and in otitis media in children. They can also cause sinusitis, bronchitis, bacteraemia, meningitis and other infectious processes.

Pneumococci were first noticed in 1881 by Pasteur and Sternberg independently. They produced a fatal septicaemia in rabbits by inoculating human saliva and isolated pneumococci from the blood of the animals. But the relation between pneumococci and pneumonia was established only later by Fraenkel and Weichselbaum independently in 1886.

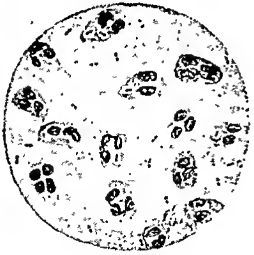

Fig 24.1 Pneumococci in pus.

Morphology: Pneumococci are typically small (1μ), slightly elongated cocci, with one end broad or rounded and the other pointed, presenting a flame shaped or lanceolate appearance. They occur in pairs (diplococci), with the broad ends in apposition, the long axis of the coccus parallel to the line joining the two cocci in a pair. They are capsulated; the capsule enclosing each pair. Capsules are best seen in material taken directly from exudates and may be lost on repeated cultivation. In cultures, the typical morphology may not be apparent and the cocci are more rounded, tending to occur in short chains. They are nonmotile and nonsporing.

They are readily stained with aniline dyes and are Gram positive. The capsule may be demonstrated as a clear halo in Indian ink preparations or may be stained directly by special techniques.

Cultural characteristics: Pneumococci have complex growth requirements and grow only in enriched media. They are aerobes and facultative anaerobes, the optimum temperature being 37°C (range 25°C–42°C) and pH 7.8 (range 6.5–8.3). Growth is improved by 5–10 per cent CO_2.

On blood agar, after incubation for 18 hours, the colonies are small (0.5–1 mm), dome shaped and glistening, with an area of greenish discolouration (alpha haemolysis) around them.

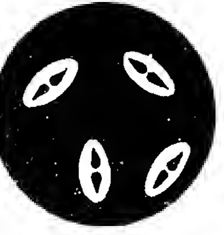

Fig. 24.2 Pneumococci. Indian ink preparation to show capsules

On further incubation, the colonies become flat, with raised edges and central umbonation, so that concentric rings are seen when viewed from above (draughtsman appearance). Some strains that produce abundant capsular material (type 3) form large, mucoid colonies (*Diplococcus mucosus*).

Under anaerobic conditions colonies on blood agar are surrounded by a zone of beta haemolysis due to oxygen labile pneumolysin O. In liquid media such as glucose broth, growth occurs as uniform turbidity. The cocci readily undergo autolysis in cultures, due to the activity of intracellular enzymes. Autolysis is enhanced by bile salts, sodium lauryl sulphate and other surface active agents. Heat killed cultures do not undergo autolysis.

Biochemical reactions: Pneumococci ferment several sugars, forming acid only. Fermentation is tested in Hiss's serum water or serum agar slopes. Fermentation of inulin by pneumococci is a useful test for differentiating them from streptococci as the latter do not ferment it.

Pneumococci are bile soluble. If a few drops of 10% sodium deoxycholate solution are added to 1.0 ml of an overnight broth culture, the culture clears due to the lysis of the cocci. Alternatively, if a loopful of 10% deoxycholate solution is placed on a pneumococcus colony on blood agar the colony lyses within a few minutes. Bile solubility is a constant property of pneumococci and hence is of diagnostic importance. The bile solubility test is based on the presence in the pneumococci of an autolytic amidase that cleaves the bond between alanine and muramic acid in the peptidoglycan. The amidase is activated by surface active agents such as bile or bile salts, resulting in lysis of the organisms. The test should be carried out at neutral pH using deoxycholate and live young cells in saline suspension.

Pneumococci are catalase and oxidase negative.

Resistance: Pneumococci are delicate organisms and are readily destroyed by heat (thermal death point 52°C for 15 minutes) and antiseptics. In cultures, they die on prolonged incubation, perhaps due to an accumulation of toxic peroxides. Strains may be maintained on semisolid blood agar or by lyophilisation.

They are sensitive to sulphonamides and several antibiotics. Resistance may occur to sul-

Fig. 24.3 Draughtsman appearance of pneumococcus colonies. Left—view from above to show concentric rings. Right—side view in cross section

phonamides, less often to tetracyclines and very rarely to penicillin. Till 1967 almost all strains were sensitive to less than 0.05 μ g/ml of penicillin. Subsequently increasingly resistant strains began appearing in Australia followed by other parts of the World. In 1977, South African strains resistant to 2–10 μ g/ml appeared. Resistance to tetracyclines and a few other antibiotics were noticed earlier, Multiple resistant strains have also been reported.

The sensitivity of pneumococci to optochin (ethyl hydrocuprein) 1/500,000 is useful in differentiating them from streptococci. When a disc impregnated with optochin is applied on a plate of blood agar inoculated with pneumococci, a wide zone of inhibition appears on incubation.

Antigenic properties: The most important antigen of the pneumococcus is the type specific capsular polysaccharide. As this polysaccharide diffuses into the culture medium or infective exudates and tissues, it is also called the 'specific soluble substance' (SSS). Pneumococci are classified into types based on the antigenic nature of the capsular polysaccharide. Pneumococci isolated from lobar pneumonia were originally classified into three types, I, II and III, and a heterogeneous group IV. Members of group IV were later classified into types and now more than 85 different serotypes are recognised, named 1,2,3,etc.

Typing may be carried out by 1) agglutination of the cocci with the type specific antiserum, 2) *precipitation* of the SSS with the specific serum or. 3) by the capsule swelling reaction described by Neufeld (1902). In the capsule swelling or 'quellung' reaction (Quellung = swelling), a suspension of pneumococci is mixed on a slide with a drop of the type specific antiserum and a loopful of methylene blue solution. In presence of the homologous antiserum, the capsule becomes apparently swollen, sharply delineated and refractile. The quellung test can be done directly with sputum from acute pneumonia cases, and used to be a routine bedside procedure in former days when the specific antiserum was used for the treatment of pneumonia.

The antigenicity of the capsular polysaccharide varies in different species. It is antigenic in man and rabbits. But in mice, large doses (500 μg) induce no immunological response (immunological paralysis), while small doses (0.5 μg) are antigenic.

Pneumococci contain other antigens also — a nucleoprotein deep inside the cell and a somatic 'C' carbohydrate antigen, both of which are species specific, and an M protein which is characteristic for each type. Antibodies to these antigens are not protective. Another major antigenic component of pneumococcus is the Forssman antigen which is a lipoteichoic acid.

An abnormal protein (beta globulin) that precipitates with the somatic 'C' antigen of pneumococci, appears in the acute phase sera of cases of pneumonia but disappears during convalescence. It also occurs in some other pathological conditions. This is known as the 'C-reactive protein' (CRP). Its apparent antibody-like relation to the 'C' antigen of pneumococcus is only fortuitous. It is not an antibody produced as a result of pneumococcal infection. It is an 'acute phase' substance, produced in hepatocytes. Its production is stimulated by bacterial infections, inflammation, malignancies and tissue destruction. It disappears when the inflammatory reactions subside. CRP is used as an index of respone to treatment in rheumatic fever and certain other conditions. CRP is tested by capillary precipitation of the patients' sera with antisera prepared in rabbits against purified CRP, or by passive agglutination using latex particles coated with anti-CRP antibody.

Variation: On repeated subculture, pneumococci undergo a smooth-to-rough (S-R) variation. In the R form, the colonies are rough and the cocci are noncapsulated, autoagglutinable and avirulent. R forms arise as spontaneous mutants and outgrow the parental S forms in artificial culture; in tissues, such R mutants are eliminated by phagocytosis.

Rough pneumococci derived from capsulated cells of one serotype may be made to produce

capsules of the same or different serotypes, on treatment with DNA from the respective serotypes of pneumococci. This transformation, which may be demonstrated *in vivo* or *in vitro*, was discovered by Griffith (1928) and is of considerable historical interest as the first instance of genetic exchange of information in bacteria.

Toxins and other virulence factors: Pneumococci produce an oxygen labile haemolysin and a leucocidin, but these are weak and make no contribution to virulence. The virulence of pneumococci is directly dependent on the production of the capsular polysaccharide. This substance, because of its acidic and hydrophilic properties, protects the cocci from phagocytosis. Capsulated pneumococci are not phagocytosed efficiently in fluid media or exudates. They are, however, susceptible to 'surface phagocytosis', being engulfed against a firm surface, such as fibrin clot or epithelium.

The enhanced virulence of type 3 pneumococcus is due to the abundance of its capsular material. Noncapsulated strains are avirulent. The antibody to the capsular polysaccharide affords protection against infection.

Pathogenicity: Experimentally, fatal infection can be produced in mice or rabbits by intraperitoneal inoculation of pneumococci. Death occurs in one to three days and pneumococci can be demonstrated in large numbers in the peritoneal exudate and heart blood.

In man, over 80 per cent of lobar pneumonia and 60 per cent of bronchopneumonia are caused by pneumococci. They also produce suppurative infections in various other parts of the body.

1. Lobar pneumonia does not always follow infection with a virulent pneumococcus. Airborne infection of the respiratory tract with pneumococcus is a frequent occurrence. The cocci are usually eliminated by the natural defence mechanisms, or they establish symptomless carriage in the throat. Pneumonia results only when, in such a carrier, the general resistance is lowered. The pneumococci penetrate the bronchial mucosa and spread through the lung along the peribronchial tissues and lymphatics. Bacteraemia is frequent during the early stage of lobar pneumonia and bears a relation to the severity and outcome of the disease. The toxaemia is due to the diffusion of the capsular polysaccharide into the blood and tissues. The fall of temperature by 'crisis' and the relief of toxic symptoms coincide with the complete neutralisation of SSS by the anticapsular antibody.

In adults, types 1–8 are responsible for about 75 per cent of cases of pneumococcal pneumonia and for more than 50 per cent of all fatalities to pneumococcal bacteraemia. In children, types 6, 14, 19 and 23 are frequent causes.

2. Bronchopneumonia is almost always a secondary infection and commonly follows viral infections of the respiratory tract. Pneumococcus is the most common aetiological agent. This may be caused by any serotype of pneumococcus. The damage to respiratory epithelium and excessive bronchial secretions caused by the primary infection facilitate the invasion of pneumococci along the bronchial tree. Other causative agents include *Staph. aureus*, *Klebsiella pneumoniae*, *Str. pyogenes*, *H. influenzae*, *Ps. aeruginosa*, *Proteus species*, *Serratia marcescens*, anaerobic organisms such as *Peptostreptococcus*, *Peptococcus*, *Fusobacterium* and *Bacteroides*. Bronchopneumonia is frequently a terminal event in aged and debilitated patients.

3. Pneumococci are commonly associated with the acute exacerbations in chronic bronchitis. The copious respiratory secretions in chronic bronchitis aid pneumococcal invasion. Another bacterium commonly associated with this condition is *Haemophilus influenzae*.

4. Meningitis is the most serious of pneumococcal infections. It is usually secondary to other pneumococcal infections such as pneumonia, otitis media, sinusitis or conjunctivitis, but in a proportion of casess, other foci of infection may not be demonstrable. The disease is commoner in children. Untreated cases are almost invariably fatal. Even with antibiotic therapy, the case fatality rate is about 25 per cent.

5. Pneumococci may also produce suppurative lesions in other parts of the body — empyema, pericarditis, otitis media, sinusitis, conjunctivitis, suppurative arthritis and peritonitis, usually as complications of pneumonia.

Epidemiology: Natural infection with pneumococci has been reported in some species of animals such as guinea pigs, but they have little relation to human disease. The source of human infection is the respiratory tract of carriers, and less often, of patients. Pneumococci occur in the throat of approximately half the population sampled at any one time. They are transmitted from one to another by an inhalation of contaminated dust, droplets or droplet nuclei. Dissemination is facilitated by crowding.

Infection usually leads only to temporary pharyngeal carriage. Disease results only when the host resistance is lowered by contributory factors such as respiratory viral infections, pulmonary congestion, stress, malnutrition or alcoholism.

Pneumococcal serotypes vary greatly in virulence. The case fatality rates of pneumonia may vary according to the virulence of the infecting serotype. Type 3 is the most virulent, with a case fatality of 40–60 per cent.

Lobar pneumonia is usually a sporadic disease but epidemics may occur among closed communities as in army camps. The incidence of bronchopneumonia increases when an epidemic of influenza or other viral infections of the respiratory tract occurs. Cases are more common in winter and affect the two extreme age groups more often.

Laboratory diagnosis: The clinical diagnosis of pneumonia is easy, but as the disease may be caused by several different microorganisms, aetiological diagnosis should be made by laboratory tests. This is of great importance in treatment.

In the acute phase of lobar pneumonia, the rusty sputum contains pneumococci in large numbers, with hardly any other kind of bacterium. They may be demonstrated by Gram stain and typed by the quellung test in wet films — a procedure that was routine when antisera were employed for treatment. In later stages of the disease, pneumococci are less abundant.

The sputum, after homogenisation if necessary, is inoculated on blood agar plates and incubated at 37°C under 5–10 per cent CO_2. Growth occurs after overnight incubation. Where sputum is not available, as in infants, serum-coated laryngeal swabs may be used for culture.

The isolate of pneumococcus may be typed with appropriate antisera. Besides yielding epidemiological information, typing is also of prognostic significance. But routine typing is not necessary, nor is it possible as typing sera are not available.

TABLE 24.1
Differentiation between Pneumococcus and Str. viridans

	Pneumococcus	*Str. viridans*
1. Morphology	Capsulated, lanceolate diplococci	Noncapsulated, oval or round cells in chains
2. Quellung test	Positive	Negative
3. Colonies	Initially dome shaped later, 'draughtsman' colonies	Dome shaped
4. Growth in liquid media	Uniform turbidity	Granular turbidity, powdery deposit
5. Bile solubility	Invariably positive	Invariably negative
6. Inulin fermentation	Positive	Negative
7. Optochin sensitivity	Positive	Negative
8. Intraperitoneal inoculation in mice	Fatal infection	Nonpathogenic

From specimens where pneumococci are expected to be scanty, isolation may be obtained by intraperitoneal inoculation in mice, even if cultures are negative. Inoculated mice die in one to three days and pneumococci may be demonstrated in the peritoneal exudate and heart blood. The test may be negative with occasional strains that are avirulent for mice (type 14 strains).

In the acute stage of pneumonia, the organism may be obtained from blood culture in glucose broth. Isolation of pneumococci from blood indicates bad prognosis.

In case of meningitis, presumptive diagnosis may be made from Gram stained films of CSF. Gram positive diplococci can be seen both inside polymorphs as well as extracellularly. Diagnosis is confirmed by culture. In cases negative by culture, it may be possible to establish the diagnosis by demonstrating the SSS in CSF by precipitation with antisera.

Capsular polysaccharide can be demonstrated in blood, urine and cerebrospinal fluid by counterimmunoelectrophoresis. Antibodies can be demonstrated by agglutination, quantitative precipitation, mouse protection tests and bactericidal tests with whole blood. Indirect haemagglutination, indirect FA test and radioimmunoassay have been employed.

Prophylaxis: Immunity is type specific and associated with antibody to the capsular polysaccharide. The existence of more than 85 serotypes would seem to make prophylactic immunisation impracticable. But, as most pneumonia cases are caused by a limited number of serotypes, vaccination is possible. Trials during World War II have shown that vaccination with capsular polysaccharide from the prevalent serotypes would confer a high degree of protection. In 1983 a polysaccharide vaccine containing 23 types was licensed in the U.S.A. Such vaccines are used specially in children who are above two years of age and may be used in elderly, debilitated or immunosuppressed individuals. It is contraindicated in patients with lymphomas.

Further Reading

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25 Neisseria

The family *Neisseriacea* includes *Neisseria* species and *Branhamella catarrhalis* as well as *Acinetobacter* and *Kingella* and *Moraxella* species.

The genus Neisseria consists of Gram negative, aerobic, nonsporulating, nonmotile, oxidase positive cocci typically arranged in pairs (diplococci). Besides the two important pathogens, *N. meningitidis* and *N. gonorrhoeae*, the genus contains about 30 other species that occur as commensals in the mouth or upper respiratory tract.

Gonococci and meningococci are closely related with 70 per cent DNA homology, and are differentiated by a few laboratory tests and specific characteristics. Meningococci have polysaccharide capsules while gonococci do not. Meningococci rarely have plasmids while most gonococci do.

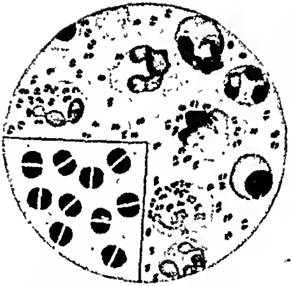

Fig 25.1 Meningococcus in cerebrospinal fluid Inset—enlarged view to show flat adjacent sides of the cocci

Neisseria meningitidis (*Meningococcus*; *Diplococcus intracellularis meningitidis*)

N. meningitidis causes meningococcal meningitis or cerebrospinal fever which may occur sporadically or as epidemics. The meningococcus was first described and isolated in 1887 by Weichselbaum from the spinal fluid of a patient.

Morphology: They are Gram negative, oval or spherical cocci, 0.6–0.8 μ in size, typically arrranged in pairs with the adjacent sides flattened (Fig. 25.1). The long axis of the coccus is at right angles to a line joining the two cocci in a pair. Considerable variations occur in size, shape and staining properties, especially in older cultures, due to autolysis. In smears from lesions, the cocci are more regular and generally intracellular. They are nonmotile. Most fresh isolates are capsulated.

Cultural characteristics: Meningococci have exacting growth requirements and do not grow on ordinary media. Growth occurs on media enriched with blood, serum or ascitic fluid. These substances are believed to promote growth by neutralising certain inhibiting substances found in culture media rather than by providing additional nutritional needs.

They are strict aerobes, no growth occurring anaerobically. The optimum temperature for growth is 35°C–36°C. No growth takes place

below 30°C. Optimum pH is 7.4–7.6. Growth is facilitated by 5–10 per cent CO_2 and high humidity.

On solid media, after incubation for 24 hours, the colonies are small (about 1 mm in diameter), translucent, round, convex, bluish grey, with a smooth glistening surface and with entire edges. The colonies are typically lenticular in shape, butyrous in consistency and easily emulsifiable. Weak haemolysis occurs on blood agar. Smooth and rough types of colonies are found. Growth is poor in liquid media, producing a granular turbidity with little or no surface growth.

Blood agar, chocolate agar and Mueller-Hinton starch-casein hydrolysate-agar are the media commonly used for culturing meningococci. Modified Thayer-Martin (with Vancomycin, Colistin and Nystatin), Martin Lewis and New York City media are other media used.

Biochemical reaction: They are catalase and oxidase positive. The prompt oxidase reaction helps the identification of neisseria (both meningococcus and gonococcus) in mixed cultures. When freshly prepared 1% solution of oxidase reagent (tetramethyl paraphenylene diamine hydrochloride) is poured on the culture plate, neisseria colonies turn deep purple. Subcultures should be made immediately, as the organism dies on prolonged exposure to the reagent. The test may also be performed by rubbing a little of the growth with a loop on a strip of filter paper moistened with the oxidase reagent (Kovacs' method). A deep purple colour appears immediately.

Indole and hydrogen sulphide are not produced and nitrates are not reduced. Glucose and maltose are fermented, producing acid, but no gas (gonococci ferment glucose, but not maltose). Fermentation is usually tested on peptone-serum-agar slopes containing the sugar and indicator.

Antigenic properties and classification

At least 13 serogroups of meningococci — A, B, C, D, X, Y, Z, W–135, 29E, H, I, K, and L — have been identified on the basis of immunological specificity of the capsular polysaccharides. Outbreaks and sporadic disease in the Western hemisphere in the last decade have been caused mainly by groups B, C, W–135, Y; outbreaks in Southern Finland and Sao Paulo, Brazil, were due to groups A and C, and, especially, group A are associated with epidemic disease.

On the basis of molecular weight, the outer membrane proteins of meningococci have been divided into five classes. All strains have either class 2 or class 3 proteins. These are analogous to the protein I of gonococci and are responsible for the serotype specificity of meningococci. As many as 20 serotypes have been defined. Serotypes 2 and 15 have been associated with epidemic disease. Meningococci are piliated but, unlike gonococci, they do not form distinctive colony types indicating piliated bacteria. Meningococcal LPS is responsible for many of the toxic effects in meningococcal disease.

Isolation and identification of specific polysaccharide antigens have enabled the production of vaccines. Vaccines that contain groups A and C polysaccharides are licensed for USA and several other countries. They have been found efficient against infections in older children and adults due to these serogroups. The group A vaccine is effective in controlling outbreaks in children beyond their first year. There is some evidence that they are protective when administered as early as three months. The group C vaccines while effective in children over two years of age is poorly immunogenic and apparently not protective in younger children. But about 30 per cent of group C cases are in children less than two years of age. Serogroups Y and W-135 vaccines have been found to induce significant bactericidal response and are in use in selected groups in situations such as military and civilian epidemics. Serogroup B which causes significant disease has no satisfactory vaccine.

Besides capsular antigens, meningococci possess carbohydrate and protein somatic antigens. These have not been fully characterised.

Resistance: Meningococci are very delicate organisms, being highly susceptible to heat, desiccation, alterations in pH and to disinfectants. They are susceptible to sulphonamides, penicillin, streptomycin and many other antibiotics. But strains resistant to sulphonamides are now common. Penicillin resistant strains first noticed in the Philippines and West Africa in 1976 have now been reported from several countries.

Pathogenicity: Cerebrospinal meningitis and meningococcal septicaemia are the two types of meningococcal disease. Meningococci are strict human parasites, gaining entry into the body via the nasopharynx. Infection is usually asymptomatic. In some, local inflammation ensues, with rhinitis and pharyngitis. Dissemination occurs only in a small proportion.

The manner in which the cocci spread from the nasopharynx to the meninges is controversial. The spread may be directly along the perineural sheath of the olfactory nerve, through the cribriform plate to the subarachnoid space, or much more probably, through the bloodstream. It is also believed that in certain cases, the site of entry of the meningococcus may be the conjunctiva. Cases of meningococcal purulent conjunctivitis have been reported. On reaching the central nervous system, a suppurative lesion of the meninges is set up, involving the surface of the spinal cord as well as the base and cortex of the brain. The cocci are invariably found in the spinal fluid, both free and within the leucocytes. Case fatality is variable, but in untreated cases may be as high as 80 per cent. Survivors may have sequelae such as blindness and deafness. Some cases develop chronic or recurrent meningitis.

Meningococcaemia presents as acute fever with chills, malaise and prostration. Haemorrhagic manifestations are characteristic. A typical petechial rash involving skin and mucosa occurs early in the disease. Meningococci may be isolated from the petechial lesions. Metastatic involvement of the joints, ears, eyes, lungs and adrenals may occur. Neisseria bacteraemia is favoured by complement deficiency (C_5, C_6, C_7 or C_8).

The pathogenic agent in meningococcal disease appears to be the endotoxin, released by autolysis. The vascular endothelium is particularly sensitive to the endotoxin and the haemorrhagic manifestations may be due to a Shwartzman-like phenomenon. In fulminating meningococcal septicaemia (Waterhouse-Friderichsen syndrome), there occur adrenal haemorrhage and profound shock.

Natural infection is limited to man. Intraspinal inoculation of large numbers of cocci may produce a picture of meningitis in monkeys. Intraperitoneal inoculation of the cocci suspended in hog gastric mucin brings about a fatal infection in mice.

Epidemiology: The human nasopharynx is the only reservoir of the meningococcus. Asymptomatic nasopharyngeal carriers rarely contract the illness; they serve to infect their contacts. Transmission is essentially by airborne droplets, or less often by fomites. During interepidemic periods, the carrier rate is about 5–10 per cent. An increase in carrier rate heralds the onset of an epidemic. During epidemics, carrier rates in closed communities may go up to 90 per cent. Meningitis is more common in children below the age of five years and in males. Epidemics usually occur in semiclosed communities that live crowded together, as in jails and ships formerly, and in army camps in recent times. Group B strains have accounted for most cases of meningitis in the U.K. in recent years. Elsewhere, groups A and C have been mainly active. Epidemic meningitis caused by group A meningococcus affected Delhi and surrounding areas in early 1985.

Laboratory diagnosis: As purulent meningitis may be produced by other bacteria also (commonly pneumococci and *Haemophilus influenzae;* less commonly several other bacteria), it is necessary to establish the specific aetiology for ensuring proper treatment. In cerebrospinal meningitis, meningococci are present in large

numbers in the spinal fluid, and in the early stage, in the blood also. Demonstration of meningococci in the nasopharynx helps in detection of carriers.

1. Examination of CSF: The fluid will be under pressure and turbid, with large numbers of pus cells. For bacteriological examination, if sufficient quantity is available, the CSF is divided into three portions. One portion is centrifuged and Gram stained smears are prepared from the deposit. Meningococci will be seen, mainly inside polymorphs, but often extracellularly also. When present in small numbers, meningococci may be detected by immunofluorescent techniques. The supernatant will contain meningococcal antigen, which may be demonstrated by a precipitation test with polyvalent or monovalent antimeningococcal serum. Counterimmunoelectrophoresis provides a rapid and sensitive method for demonstration of meningococcal antigen. The second portion of the CSF is inoculated on blood agar or chocolate agar plates and incubated at 35°C to 36°C under 5–10 per cent CO_2. Colonies appear after 18–24 hours and may be identified by morphology and biochemical reactions. It is important to remember that morphologically similar organisms such as *N. flavescens*, *N. flava* and *Mima polymorpha* may also cause purulent meningitis occasionally. The meningococcus isolated may be grouped, if required, by agglutination with the appropriate sera. The third portion of CSF is incubated overnight, either as such or after adding an equal volume of glucose broth and then subcultured on chocolate agar. This method may sometimes succeed where direct plating fails. Isolation may also be obtained by inoculation into the yolk sac of eight to ten day old embryonated eggs.

A presumptive diagnosis can usually be made by microscopic examination of stained smears of CSF. Meningococci may be scanty or undetectable in late or treated cases.

2. Blood culture: In meningococcaemia and in early cases of meningitis, blood culture is often positive. Cultures should be incubated for 4–7 days, with daily subcultures. Meningococcal antigen can be found in the blood in active disease.

3. Nasopharyngeal swab: This is useful for detection of carriers. Sampling should be done without contamination with saliva. This is achieved by the use of the West's postnasal swab. The swab should be held in a suitable transport medium (e.g., Stuart's) till it is plated.

4. Petechial lesions: Meningococci may sometimes be demonstrated in petechial lesions by microscopy and culture.

5. Autopsy: At autopsy, specimens may be collected from the meninges, lateral ventricles or the surface of the brain and spinal cord for smear and culture. Meningococci may die if specimens are not collected within twelve hours of the death of the patient.

6. Retrospective evidence: Retrospective evidence of meningococcal infection may be obtained by demonstrating complement fixing antibodies in convalescent sera. Passive haemagglutination and radioactive antigen binding tests are much more sensitive techniques for detection of antibodies.

Treatment

Sulphonamides formed the mainstay in treatment formerly, but now resistance to them has become common. Meningococci are uniformly sensitive to penicillin, with MIC of about 0.3 mcg/ml. Penicillin G in high doses, given intravenously or intrathecally if necessary, is the treatment of choice. Prompt treatment has reduced the case fatality to about five to ten per cent. Chloramphenicol is used in persons allergic to penicillins.

Prophylaxis

Sulphonamides were used widely and successfully for chemoprophylaxis formerly, but the emergence of resistant strains has curtailed their efficacy. Penicillin is unable to eradicate the carrier state. Rifampin or minocycline are recommended for chemoprophylaxis at present. The

limitations are development of resistance to rifampin and undesirable side effects for minocycline.

Use of meningococcal vaccines for prophylaxis has been dealt with under 'antigenic properties'.

Neisseria gonorrhoeae *(Gonococcus)*

N. gonorrhoeae causes the venereal disease gonorrhoea. The gonococcus was first described in gonorrhoeal pus by Neisser in 1879. Bumm in 1885 cultured the coccus and proved its pathogenicity by inoculating human volunteers. Gonococci resemble meningococci very closely in many properties.

Morphology: In smears from urethral discharge in acute gonorrhoea, the organism appears as a diplococcus with the adjacent sides concave, being typically reniform or pear shaped. It is found *predominantly within the polymorphs*, some cells containing as many as a hundred cocci.

Gonococci possess pili on their surface. Pili facilitate adhesion of the cocci to mucosal surfaces and promote virulence by inhibiting phagocytosis. Piliated gonococci agglutinate human red blood cells but not red cells from other mammals. The haemagglutination is not inhibited by mannose.

Cultural characteristics: Gonococci are more difficult to grow than meningococci. They are aerobic, but may grow anaerobically also. Growth occurs best at pH 7.2–7.6 and at a temperature of 35°C–36°C. It is essential to provide 5–10 per cent CO_2. They grow well on chocolate agar. A popular selective medium is the Thayer-Martin medium (containing vancomycin, colistin and nystatin) which inhibits most contaminants including nonpathogenic neisseria. The Chacko-Nair egg enriched medium, although not as selective, also supports good growth.

Colonies are small, round, translucent, convex or slightly umbonate, with finely granular surface and lobate margins. They are soft and easily emulsifiable.

Four types of colonies have been recognised, T1 to T4. Types 1 and 2 form small brown colonies. The cocci are piliated, auto-agglutinable and virulent. Types 3 and 4 form larger, granular, nonpigmented colonies. The cocci are non-piliated, form smooth suspensions and are avirulent. Fresh isolates from acute cases of gonorrhoea generally form T1 and T2 colonies. On serial subculture they change to T3 or T4 colonial morphology. T1 and T2 types are also known as P+ and P++, respectively, while T3 and T4 are known as P−.

Biochemical reactions: Gonococci resemble meningococci except in the fermentation of maltose. Gonococci ferment only glucose and not maltose.

Antigenic properties

Gonococci are antigenically heterogenous. They are capable of changing their surface structures *in vitro*. They probably do so *in vivo* as well, to avoid host defences. The surface structures include the following:

1. Pili: They are several micrometers in length and are hair-like. They enhance attachment to host cells and resistance to phagocytosis. They are made up of stacked pilin proteins. The pilins of almost all strains are antigenically different and a single strain can make several antigenically different forms of pilin.

2. Protein I: It extends through the gonococcal cell membrane. It forms pores in the surface. Each strain expresses only one type of Protein I but the Protein I of different strains is antigenically different. Serological typing of Protein I by agglutination reaction using monoclonal antibodies has demonstrated 18 serovars of type 1A and 28 serovars of type IB.

3. Protein II: This protein takes part in adhesion of gonococci within colonies and in its attachment to the host cells. One part of it is in the gonococcal outer membrane and the rest is exposed on the surface. A strain of gonococcus can express 0–2 or occasionally three types of Protein II. Protein II is present in gonococci from opaque colonies. It may be present or absent from those in transparent colonies.

4. Protein III: This associates with Protein I in the formation of pores on the cell surface.

5. Lipopolysaccharide: Toxicity in gonococcal infections is largely due to the endotoxic effects of this.

6. Other proteins: Several proteins of poorly defined roles in pathogenicity have been described. Gonococci as well as meningococci elaborate an IgA1 protease that splits and inactivates IgA1.

Resistance: The gonococcus is a very delicate organism, readily killed by heat, drying and antiseptics. It is a strict parasite and dies in 1–2 hours in exudates outside the body. Formerly, it was highly susceptible to sulphonamides, penicillin and many other antibiotics. But gonococci have steadily developed resistance to one antibiotic after another.

In cultures, the coccus dies in 3–4 days, but survives in slant cultures at 35°C if kept under sterile paraffin oil. Cultures may be stored for years if frozen quickly and left at −70°C.

Pathogenicity: Gonorrhoea is a venereal disease which has been known from ancient times. The name gonorrhoea (meaning, flow of seed) was first employed by Galen in 130 A.D.

The disease is acquired by sexual contact. The first step in infection is adhesion of gonococci to urethra or other mucosal surfaces. Pili are involved in this adhesion. Adhesion is rapid and firm so that micturition after exposure offers no protection against infection. The cocci penetrate through intercellular spaces and reach the subepithelial connective tissue by the third day after infection. The incubation period is 2–8 days. In males, the disease starts as an acute urethritis with a mucopurulent discharge containing gonococci in large numbers. The infection extends along the urethra to the prostate, seminal vesicles and epididymis. Chronic urethritis may lead to stricture formation. The infection may spread to periurethral tissues, causing abscesses and multiple discharging sinuses ('watercan perineum').

In females, the initial infection involves the urethra and cervix uteri. The vaginal mucosa is not usually affected in adults, because the stratified squamous epithelium is resistant to infection by the cocci and also because of the acid pH of the vaginal secretions. (Vulvovaginitis occurs in prepubertal girls.) The infection may extend to the Bartholin's glands, endometrium and Fallopian tubes. Salpingitis may lead to sterility. Clinical disease is as a rule less severe in females, many of whom may carry gonococci in the cervix without developing any clinical symptoms. Asymptomatic carriage of gonococci may also occur in males, but it is much less than in females.

Proctitis occurs in both sexes. It may develop by direct contiguous spread in the female, but in the male is usually the result of homosexual activity. Gonococcal pharyngitis has been reported rarely and may follow abnormal sexual practices. Conjunctivitis may occur, usually due to autoinoculation by fingers. Blood invasion may occur from the primary site of infection and may lead to metastatic lesions such as arthritis, ulcerative endocarditis and very rarely meningitis. Occasionally cases of pyaemia have been reported.

A nonvenereal infection is gonococcal ophthalmia in the newborn, which results from direct

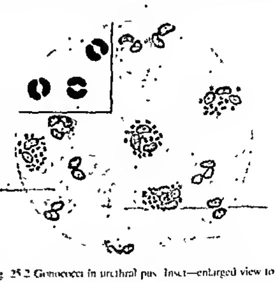

Fig. 25.2 Gonococci in urethral pus. Inset—enlarged view to show kidney shaped cells with adjacent surfaces concave

infection during passage through the birth canal. Gonococcal bacteraemia leads to skin lesions, especially haemorrhagic papules and pustules on the hands, forearms, feet and legs and to tenosynovitis and suppurative arthritis, usually of the knees, ankles and wrists.

Gonococci contain serveral plasmids. Ninety five per cent of the strains have a small 'cryptic' plasmid of unknown function. Two other plasmids contain genes that code for beta lactamase production which causes resistance to penicillin. These plasmids are transmissible by conjugation among gonococci and are similar to the plasmids found in the penicillinase producing *Haemophilus* and may have been acquired from them or other Gram negative organisms. About 5–20 per cent of gonococci contain a plasmid with the genes that code for conjugation, the incidence being highest in geographic areas where penicillinase producing gonococci are most common.

Epidemiology: Gonorrhoea is an exclusively human disease, there being no natural infection in animals. Experimental disease may be produced in chimpanzees by urethral inoculation. A lethal Infection can be produced in mice by intracerebral inoculation.

The only source of infection is a human patient or carrier. The existence of asymptomatic carriage in females makes them a reservoir serving to perpetuate infection among their male contacts. The mode of infection is almost exclusively venereal. Fomites do not play any significant role as the cocci die rapidly outside the human body. The only nonvenereal infection is ophthalmia neonatorum. Once so common, this has now been controlled by the practice of instilling silver nitrate solution into the eyes of all newborn babies (Crede's method).

When sulphonamides and, later, penicillin were found very effective for treatment of gonorrhoea, it was hoped that the disease could be eradicated. But after a temporary decline, the incidence of the disease has been rising steeply. In 1970, the global incidence of new cases was estimated at 16 million, making it one of the commonest of infectious diseases. In some areas, gonorrhoea has reached epidemic proportions, especially in adolescents and young adults. The reasons for the increase in gonorrhoeal infection are largely social and cultural. A higher incidence of gonorrhoea has been observed in persons belonging to blood group B. The basis for this is not known.

Laboratory diagnosis: In the acute stage, diagnosis can be established readily, but chronic cases sometimes present great difficulties. In acute gonorrhoea, the urethral discharge contains gonococci in large numbers. The meatus is cleaned with a gauze soaked in saline and a sample of the discharge collected with a platinum loop for culture, or directly on slides for smears. In females, besides the urethral discharge, cervical swabs also should be collected. This should be done carefully, using a speculum. High vaginal swab is not satisfactory.

In chronic infections, there may not be any urethral discharge. The 'morning drop' of secretion may be examined or some exudate may be obtained after prostatic massage. It may also be possible to demonstrate gonococci in the centrifuged deposits of urine in cases where no uretheral discharge is available.

The demonstration of intracellular Gram negative diplococci in stained smears provides a presumptive evidence of gonorrhoea in the male. It has to be emphasised that diagnosis of gonorrhoea by smear examination is unreliable in females as some of the normal genital flora have an essentially similar morphology. The use of fluorescent antibody techniques for the identification of gonococci in smears has increased the sensitivity and specificity of diagnosis by microscopy.

For culture, specimens should be inoculated on prewarmed plates immediately on collection. If this is not possible, specimens should be collected with charcoal impregnated swabs and sent to the laboratory in Stuart's transport medium. From acute gonorrhoea, cultures can be obtained readily on chocolate agar incubated at 35°C to 36°C

Precipity.
Passivehaem.
Gn.

TABLE 25.1

Differential characteristics of neisseriae

Species	Colonies	Growth		Fermentation			Serological classification
		On nutrient agar	At 22°C	Glucose	Maltose	Sucrose	
N. meningitidis	Round, smooth, glistening; creamy consistency	–	–	A	A	–	Eight antigenic groups
N. gonorrhoeae	Same as above, but smaller and more opalescent	–	–	A	–	–	Antigenically heterogenous
N. flavescens	Resemble meningococcus colonies but pigmented yellow	+	±	–	–	–	Antigenically distinct homogenous group
N. sicca	Small, dry, opaque, wrinkled, brittle	+	+	A	A	A	Autoagglutinable
N. catarrhalis (*Branhamella catarrhalis*)	Variable, smooth and translucent or adherent and opaque. Not easily emulsifiable	+	+	–	–	–	Autoagglutinable

under 5–10 per cent CO_2. But in chronic cases, where mixed infection is usual and in the examination of lesions such as proctitis, it is better to use a selective medium such as Thayer-Martin's medium. The growth is identified by morphology and biochemical reactions.

It may not be possible to obtain gonococci in culture from some chronic cases or from patients with metastatic lesions such as arthritis. Serological tests may be of value in such instances. The complement fixation test has been used with varying degrees of success. It becomes positive only some weeks after the infection is established and may remain positive for months or years after the disease has been cured. The test may be positive following meningococcal infections. It is necessary to use a polyvalent antigen because of the antigenic heterogeneity of gonococcal strains. The test is not suitable for routine use, but may be employed in special situations. Many other serological tests have been attempted, including precipitation, passive agglutination, immunofluorescence tests and radioimmunoassay using whole-cell lysate, pilus protein and lipopolysaccharide antigens. But no serological test has been found useful for routine diagnostic purposes.

Treatment: In 1935, when sulphonamides were introduced for treating gonorrhoea, all strains were sensitive to the drug, but resistance developed rapidly and by 1946, almost 90 per cent of the strains had become resistant in some countries. Again, when penicillin was first introduced in treatment, all strains were highly sensitive (MIC 0.005 units/ml). From 1957, strains of gonococci were noticed that were inhibited only by higher concentrations of penicillin (MIC higher than 0.1 unit/ml) and by 1970, over 50 per cent of strains from many countries were of such variety. Though resistance is not absolute, patients infected with such strains do not respond to treatment with the usual doses of penicillin. In view of this the present practice is to give very large doses of penicillin, 2.4–4.8 million units of procaine pencillin G intramuscularly, with one gram probenicid orally.

From 1976, gonococci producing B lactamase (penicillinase) have appeared. These penicillinase producing *N. gonorrhoeae* (PPNG) have spread widely (Philippines, Singapore and Sub-Saharan Africa) but their frequency is still low. Penicillin is ineffective in patients infected with such strains. They have to be treated with other agents such as tetracycline or spectinomycin.

Prophylaxis: Control of gonorrhoea consists of early detection of cases, contact tracing, health education and other general measures. As even clinical disease does not confer any immunity, vaccination has no place in prophylaxis.

Nongonococcal (nonspecific) urethritis

Along with an increase in the incidence of gonorrhoea, there has also been an increase in recent years, of cases of chronic urethritis where gonococci cannot be demonstrated. This has been called nongonococcal or nonspecific urethritis. In some of these, urethritis forms part of a syndrome consisting of conjunctivitis and arthritis in addition (Reiter's syndrome). Some of these cases may be due to gonococcal infection, the cocci persisting as L forms and hence undetectable by routine tests. But the majority of such cases are the result of infections of diverse aetiology. The most important of these are *Chlam. trachomatis* (TRIC agents), *Ureaplasma urealyticum*, *Mycoplasma hominis*. Herpes virus and cytomegalovirus also may account for some cases. Urethritis may also be caused by other bacteria (e.g., *Gardnerella vaginalis, Acinetobacter lowffii, A. nitratus*), fungi (*Candida albicans*) protozoa (*Trichomonas vaginalis*) or even by mechanical or chemical irritation. As aetiological diagnosis is seldom achieved, the management of this syndrome is difficult.

Commensal neisseriae

Several species of neisseriae inhabit the normal respiratory tract. The characteristic features of some of the common species are listed in Table

25.1 Their pathogenic significance is uncertain, though some of them (e.g. *N. flavescens, N. catarrhalis*) have been reported occasionally as having caused meningitis.

Neisseria catarrhalis is now classified as *Branhamella catarrhalis*. It is an opportunist pathogen capable of causing laryngitis, bronchopneumonia, meningitis and middle ear disease.

Further Reading

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26 Corynebacterium

Corynebacteria are Gram positive, nonacid fast, nonmotile rods with irregularly stained segments, and sometimes granules. They frequently show club shaped swellings and hence the name corynebacteria (from *coryne*, meaning club). The most important member of the genus is *C. diphtheriae*, the causative agent of diphtheria.

Diphtheria has been known from ancient times. Aretaeus, the Cappodocian, in the second century, described the Egyptian or Syriac ulcer, which most medical historians agree, can be identified as diphtheria. The disease was first recognised as a clinical entity by Bretonneau (1826) who called it 'diphtherite'. The name is derived from the tough leathery pseudomembrane formed in the disease (*diphtheros*, meaning leather). The diphtheria bacillus was first observed and described by Klebs (1883), but was first cultivated by Loeffler (1884). It is hence known as the Klebs-Loeffler bacillus. Loeffler studied the effect of the bacillus in experimental animals and concluded that the disease was due to some diffusible product of the bacillus. Roux and Yersin (1888) discovered diphtheria exotoxin and established its pathogenic effect. The antitoxin was discovered by von Behring (1890).

Corynebacterium diphtheriae

Morphology: The diphtheria bacillus is a slender rod with a tendency to clubbing at one or both ends, measuring approximately 3–$6\mu \times 0.6$–0.8μ. The bacilli are pleomorphic. They are nonsporing, noncapsulated and nonmotile. Cells often show septa, and branching is infrequently observed. They are Gram positive, but tend to be decolourised easily. Granules composed of polymetaphosphate are seen in the cells. They are more strongly Gram positive than the rest of the bacterial cell. Stained with Loeffler's methylene blue, the granules take up a bluish purple colour and hence they are called 'metachromatic' granules. They are also called volutin or Babes Ernst granules. They are often situated at the poles of the bacilli and are called polar bodies. Special stains, such as Albert's, Neisser's and Ponder's, have been devised for demonstrating the granules clearly.

The bacilli are arranged in a characteristic fashion in smears. They are usually seen in pairs or small groups, the bacilli being at various angles to each other, resembling the letters V or L. This has been called the Chinese letter or cuneiform arrangement. This is due to the incomplete separation of the daughter cells after binary fission (Fig. 26.1).

Cultural characteristics: Growth is scanty on ordinary media. Enrichment with blood, serum or egg is necessary for good growth. The optimum temperature for growth is 37°C (range 15°–40°C) and optimum pH 7.2. It is an aerobe and a facultative anaerobe.

The usual media employed for cultivation of the diphtheria bacillus are Loeffler's serum slope and tellurite blood agar. Diphtheria bacilli grow on Loeffler's serum slope very rapidly, and colonies can be seen in 6–8 hours, long before other bacteria grow. Colonies are at first small, circular, white, opaque discs, but enlarge on con-

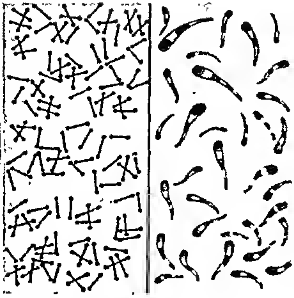

Fig. 26.1 Left: Normal forms of *C. diphtheriae*. Right: involution forms.

tinued incubation and may acquire a distinct yellow tint. Several modifications of tellurite blood agar have been utilised, such as McLeod's and Hoyle's media. Tellurite (0.04 per cent) inhibits the growth of most other bacteria, acting as a selective agent. Diphtheria bacilli reduce tellurite to metallic tellurium, which is incorporated in the colonies, giving them a grey or black colour. The growth of diphtheria bacilli may be delayed on the tellurite medium and colonies may take two days to appear. Based on colonial morphology on the tellurite medium and other properties, McLeod classified diphtheria bacilli into three types—*gravis, intermedius* and *mitis*. The names were originally proposed to relate to the clinical severity of the disease produced by the three types, gravis causing the most serious and mitis the mild variety, with intermedius being responsible for disease of intermediate severity. But this association is not constant. The necessity for typing an isolate in the laboratory has been superseded by the need to know whether the strain is toxigenic or not. But certain biological characteristics of these individual types have some value. The gravis and intermedius types are associated with a high case fatality rate, while mitis infections are less lethal. Paralytic complications are commonest in gravis, haemorrhagic complications in gravis and intermedius, and obstructive lesions in the air passages in mitis infections. In general, mitis is the predominant strain in endemic areas, while gravis and intermedius tend to be epidemic. The mitis type is better able than the more virulent types to establish a commensal relationship with the host. Wide variations have been noted in the frequency of the different types in different places at different times. There is evidence to show that the gravis and, to a less extent, the intermedius strains are able to spread more readily than the mitis in populations naturally immune or artificially immunised. Table 26.1 lists the characteristics of the three types.

The differences between the types are not always very clear cut, and it may not be possible to place some strains in any particular type. Laboratory transformation of one type to another has been reported, but in the body, the three types appear to be stable. Diphtheria bacilli ferment, with the production of acid, but no gas, glucose, galactose, maltose and dextrin, but not lactose, mannitol or sucrose. Some strains of virulent diphtheria bacilli have been found to ferment sucrose. It is necessary to employ Hiss's serum water for testing sugar fermentation.

Proteolytic activity is absent. They do not hydrolyse urea or form phosphatase, unlike some diphtheroid bacilli.

Toxin: Virulent strains of diphtheria bacilli produce a very powerful exotoxin. The pathogenic effects of the bacillus are due to the toxin. Almost all strains of gravis and intermedius (about 95–99 per cent) are toxigenic, while only about 80–85 per cent of mitis strains are so. The proportions vary with the origin of the cultures tested. Strains of all three types are invariably virulent when isolated from acute cases. Avirulent strains are common among convalescents, contacts and carriers, particularly in those with extrafaucial infection. There is considerable variation in the amount of toxin produced by the different strains, some

ABLE 26 1

Type differentiation of diphtheria bacilli

	GRAVIS	INTERMEDIUS	MITIS
Morphology	Usually short rods, with uniform staining few or no granules. Some degree of pleomorphism, with irregularly barred, snow-shoe and tear-drop forms	Long, barred forms with clubbed ends, poor granulation, very pleomorphic	Long, curved, pleomorphic rods, with prominent granules
Colony on Tellurite-blood agar	In 18 hours, colony is 1-2 mm in size, with greyish black centre, paler, semi-translucent periphery and commencing crenation of edge. In 2-3 days, 3-5 mm in size, *flat colony* with raised dark centre and crenated edge with radial striation—'daisy-head' colony	18 hour colony small, 1 mm in size, misty. Does not enlarge in 48 hrs, dull granular centre with smoother, *more glistening* periphery and a lighter ring near the edge—'frog's egg' colony	Size variable, shiny black. In 2-3 days, colony becomes flat, with a central elevation—'poached-egg' colony
Consistency of colonies	Like 'cold margarine', brittle, moves as a whole on the plate, not easily picked out or emulsifiable	Intermediate between gravis and mitis	Soft, buttery, easily emulsifiable
Haemolysis	Variable	Non-haemolytic	Usually haemolytic
Growth in broth	Surface pellicle, granular deposit, little or no turbidity	Turbidity in 24 hours, clearing in 48 hours, with fine granular sediment	Diffuse turbidity with soft pellicle later.
Glycogen and starch fermentation	Positive	Negative	Negative

strains producing the toxin abundantly and others only poorly. But the toxin produced by all strains of diphtheria bacilli is considered to be qualitatively similar. The strain almost universally used for toxin production is the 'Park-Williams 8' strain, which has been variously described as a mitis (Topley and Wilson) and an intermedius (Cruickshank) strain.

The diphtheria toxin is a protein and has been crystallised. It has a molecular weight of about 62,000. It is extremely potent and the lethal dose for a 250 g guinea pig is 0.0001 mg. It consists of two fragments A and B of M.W. 24,000 and 38,000, respectively. Both fragments are necessary for the toxic effect. When released by the bacterium, the toxin is inactive because the active site on fragment A is masked. Activation is probably accomplished by proteases present in the culture medium and infected tissues. All the enzymatic activity of the toxin is present in fragment A. Fragment B is responsible for binding the toxin to the cells. The antibody to fragment B is protective by preventing the binding of the toxin to the cells. The toxin is labile. Prolonged storage, incubation at 37°C for 4–6 weeks, treatment with 0.2–0.4 per cent formalin or acid pH converts it to toxoid. Toxoid is toxin that has lost its toxicity but not antigenicity. It is capable of producing antitoxin and reacting specifically with it.

The toxigenicity of the diphtheria bacillus depends on the presence in it of a symbiotic bacteriophage, the *beta* phage, which acts as the genetic determinant controlling toxin production. Nontoxigenic strains may be rendered toxigenic by infecting them with *beta* phage or one of its variants. This is known as lysogenic or phage conversion. The toxigenicity remains only as long as the bacillus is lysogenic. When the bacillus is cured of its phage, as by growing it in the presence of antiphage serum, it loses the toxigenic capacity.

Toxin production is also influenced by the concentration of iron in the medium. Optimum level of iron for toxin production is 0.1 mg per litre, while a concentration of 0.5 mg per litre inhibits the formation of toxin. The diphtheria toxin acts by inhibiting protein synthesis. Specially, fragment A inhibits polypeptide chain elongation in the presence of nicotinamide adenine dinucleotide by inactivating the elongation factor EF-2. It has a special affinity for certain tissues such as myocardium, adrenals and nerve endings.

Resistance: Cultures may remain viable for two or more weeks at 25°–30°C. It is readily destroyed by heat, in ten minutes at 58°C and in a minute at 100°C. It is more resistant to the action of light, desiccation and freezing than most nonsporing bacilli. It has been cultured from dried bits of pseudomembrane after 14 weeks. It remains fully virulent in blankets and floor dust for five weeks. It is easily destroyed by antiseptics. It is moderately susceptible to sulphonamides and quite susceptible to penicillin, erythromycin and the broad spectrum antibiotics.

Antigenic structure: Diphtheria bacilli are antigenically heterogeneous. By agglutination, gravis strains have been classified into 13 types, intermedius into 4 types and mitis into 40 types. Gravis strains of types I and III have been reported to be common in Great Britain, type II worldwide, type IV mainly in Egypt and type V in the U.S.A. No correlation has been established between type specificity and other characters.

Bacteriophage typing: About 35 bacteriophage types have been described. Type I to III strains are mitis, IV and VI intermedius, VII avirulent gravis and the remainder virulent gravis. The phage types are apparently stable. A system of bacteriocin (diphthericin) typing has also been described.

Pathogenicity: The incubation period in diphtheria is commonly 3–4 days, but may on occasion be as short as one day. In carriers, the incubation period may be very prolonged. The site of infection may be: 1) faucial, 2) laryngeal, 3) nasal, 4) otitic, 5) conjunctival, 6) genital — vulval, vaginal or prepucial, and 7) cutaneous — mainly around the mouth or nose; sometimes diphtheri-

tic whitlow or ulcer may occur. Faucial diphtheria is the commonest type and may vary from mild catarrhal inflammation to very widespread involvement.

According to the clinical severity, diphtheria may be classified as:

1. malignant or hypertoxic, in which there is severe toxaemia, with marked cervical adenitis (bull neck). Death is due to circulatory failure. There is high incidence of paralytic sequelae in those who recover;
2. septic, which leads to ulceration, cellulitis and even gangrene around the pseudomembrane; and
3. haemorrhagic, which is characterised by bleeding from the edge of the membrane, epistaxis, conjunctival haemorrhage, purpura and generalised bleeding tendency.

The common complications are:

1. asphyxia due to mechanical obstruction of the respiratory passage by the pseudomembrane for which an emergency tracheostomy often becomes necessary;
2. acute circulatory failure, which may be peripheral or cardiac;
3. postdiphtheritic paralysis which typically occurs in the third or fourth week of the disease; palatine and ciliary, but not pupillary, paralysis is characteristic, and spontaneous recovery is the rule; and
4. septic, such as pneumonia and otitis media.

Relapse may occur in about one per cent of cases.

Diphtheria is a toxaemia. The bacilli remain confined to the site of entry, where they multiply and form the toxin. The toxin causes local necrotic changes and the resulting fibrinous exudate, together with the disintegrating epithelial cells, leucocytes, erythrocytes and bacteria, constitute the pseudomembrane, which is characteristic of diphtheritic infection. The mechanical complications of diphtheria are due to the membrane, while the systemic effects are due to the toxin.

Diphtheria does not occur naturally in animals, but infection can be produced experimentally. Susceptibility varies in different species. Subcutaneous inoculation of a guinea pig with a culture of a virulent diphtheria bacillus will cause death in 1–4 days. At autopsy, the following features can be observed: 1) gelatinous, haemorrhagic oedema and, often, necrosis at the site of inoculation, 2) swollen and congested draining lymph nodes, 3) peritoneal exudate, which may be clear, cloudy or bloodstained, 4) congested abdominal viscera, 5) enlarged, haemorrhagic adrenals, which is the pathognomonic feature, 6) clear, cloudy or blood stained pleural exudate, and 7) sometimes, pericardial effusion.

Laboratory diagnosis: Laboratory confirmation of diphtheria is necessary for the initiation of control measures and for epidemiological purposes, but not for the treatment of individual cases. Specific treatment should be instituted immediately on suspicion of diphtheria, without waiting for laboratory tests. Any delay may be fatal.

Laboratory diagnosis consists of isolation of diphtheria bacillus and demonstration of its toxicity. One or two swabs from the lesion, collected under vision, using a tongue depressor, should be submitted to the laboratory. Diphtheria bacilli may not always be demonstrable in smears from the lesions, nor can they be confidently differentiated from some commensal corynebacteria normally found in the throat. Hence smear examination alone is not sufficient for diagnosing diphtheria but is important in identifying Vincent's angina. For this, a Gram or Leishman stained smear is examined for Vincent's spirochaetes and fusiform bacilli. It has been reported that toxigenic diphtheria bacilli may be identified in smears by immunoflourescence.

For culture, the swabs are inoculated on Loeffler's serum slope, tellurite blood agar and a plate of ordinary blood agar, the last for differentiating streptococcal or staphylococcal pharyngitis, which may simulate diphtheria. If the swab cannot be inoculated promptly it should be kept moistened with sterile horse serum so that the

bacilli will remain viable. The serum slope may show growth in 6–8 hours, but, if negative, will have to be incubated for 24 hours. Smears stained with methylene blue or one of the special stains (Neisser or Albert stain) will show the bacilli with metachromatic granules and typical arrangement. Tellurite plates will have to be incubated for at least two days before being considered negative, as growth may sometimes be delayed. The tellurite medium is particularly important in the isolation of diphtheria bacilli from convalescents, contacts and carriers as in these cases they may be outnumbered by other bacteria.

Virulence test: Any isolate of diphtheria bacillus should be tested for virulence or toxigenicity for the bacteriological diagnosis to be complete. Virulence testing may be by *in vivo* or *in vitro* methods, the former by the subcutaneous or intradermal test and the latter by the precipitation test or the tissue culture test.

Subcutaneous test: The growth from an overnight culture on Loeffler's slope is emulsified in 2–5 ml broth and 0.8 ml of the emulsion injected subcutaneously into two guinea pigs, one of which has been protected with 500 units of diphtheria antitoxin 18–24 hours previously. If the strain is virulent, the unprotected animal will die within four days, showing the autopsy appearances described earlier. This method is not usually employed as it is wasteful of animals.

Intracutaneous test: The broth emulsion of the culture is inoculated intracutaneously into two guinea pigs (or rabbits) so that each receives 0.1 ml in two different sites. One animal acts as the control and should receive 500 units of antitoxin the previous day. The other is given 50 units of antitoxin intraperitoneally four hours after the skin test, in order to prevent death. Toxigenicity is indicated by inflammatory reaction at the site of injection, progressing to necrosis in 48–72 hours in the test animal and no change in the control animal. An advantage in the intracutaneous test is that the animals do not die. As many as ten strains can be tested at a time on a rabbit.

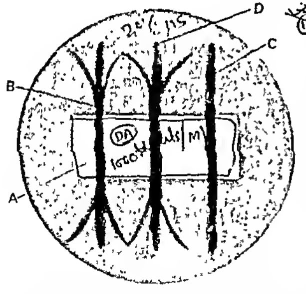

Fig. 26.2 Elek's test. A filter paperstrip impregnated with C. *diphtheriae* antitoxin. B. toxigenic strain. C. nontoxigenic strain. D. test strain showing toxigenicity.

The in vitro tests

Elek's gel precipitation test: A rectangular strip of filter paper impregnated with diphtheria antitoxin (1000 units per ml) is placed on the surface of a 20 per cent normal horse serum agar in a Petri dish while the medium is still fluid. When the agar has set, the surface is dried and narrow streaks of the test strains are made at right angles to the filter paper strip. A positive and a negative control should be put. The plate is incubated at 37°C for 24–48 hours. Toxin produced by the bacterial growth will diffuse in the agar and, where it meets the antitoxin at optimum concentration, will produce a line of precipitation (Fig. 26.2). The presence of such arrowhead lines of precipitation will indicate that the strain is toxigenic. No precipitate will form in the case of nontoxigenic strains. This test is very convenient and economical, but some brands of peptone and some samples of horse serum do not give satisfactory results.

② *Tissue culture test*: The toxigenicity of diphtheria bacilli can be demonstrated by incorporating the test strains into the agar overlay of cell culture monolayers. The toxin produced diffuses into the cells below and kills them.

Epidemiology: Diphtheria was formerly an important pediatric disease all over the world, but following the development of effective prophylactics and mass immunisation, the disease has been virtually eradicated from most advanced countries. Thus, in England and Wales, between 1915 and 1942, the number of diphtheria cases per year was about 50,000 and deaths around 2,500–4,000. It was the commonest cause of death in children aged 4–10 years. But in 1965, there were only 25 cases and no deaths. Diphtheria, however, continues to be a serious problem in the developing countries.

In endemic areas, it is mainly a disease of childhood. It is rare in the first year of life, reaches a peak between 2 and 5 years, falls slowly between 5 and 10 years and rapidly between 10 and 15 years, with only a very low incidence afterwards. Infection is rare in early infancy because of the passive immunity obtained from the mother, and in adults due to the active immunity acquired by repeated subclinical infection. The disease is commoner in rural than in urban areas. It is typically a disease of schools and institutions where children of the susceptible age are herded together. Asymptomatic carriage of the bacillus in the throat or nose is common. In endemic areas, there may be 100 carriers for every clinical case. Carriers transmit the infection to their contacts. Fomites do not seem to play an important role though in special situations toys and pencils may act as vehicles of infection. Nasal carriers harbour the bacilli for longer periods than throat carriers. In certain tropical areas, a low grade diphtheria infection of the skin is common, particularly on the lips, associated with a seropurulent nasal discharge.

In nature, diphtheria is virtually confined to man, though cows may on occasion be found to have diphtheritic infection of the udder. The infection in such cases is invariably transmitted by the milker. The infection may be spread through the milk of infected cows.

Prophylaxis: Diphtheria can be controlled by a programme of mass immunisation, as has been done in most advanced nations. The methods of immunisation available are active, passive or combined. Susceptibility to diphtheria can be detected by the Schick test. When the diphtheria toxin is injected intradermally into a susceptible person, it causes a local reaction, while in an immune individual, no reaction ensues as the toxin is neutralised by the antitoxin in circulation. This is the principle of the Schick test.

Schick test: A skin test dose of diphtheria toxin (0.2 ml containing 1/50 MLD) is injected intradermally on the left forearm and a similar dose of toxin inactivated by heating at 70°C for 30 minutes is injected on the right forearm. Readings are taken after 1, 4 and 7 days. Four types of reaction may occur.

1. Positive reaction: An area of erythema and swelling at the site of injection of the toxin, appears in 24–28 hours, reaching its maximum between the fourth and seventh days, when it measures 1–5 cm and then fading with superficial scaling and persistent brownish pigmentation. The control area injected with heated toxin will show no reaction. A positive test indicates that the person is susceptible to diphtheria.

2. Negative reaction: There is absence of any reaction on either arm. It indicates that the toxin has been neutralised by the circulating antitoxin and that the person is immune to diphtheria.

3. 'Pseudo'-reaction: There is erythema occurring within 6–24 hours and disappearing completely within four days. The reaction is the same on both arms. This indicates that the person is immune to diphtheria and also that he is hypersensitive to the components of diphtheria bacilli. This reaction is usually seen in older children and adults because they have been sensitised by repeated contact with the bacillus.

4. Combined reaction: Here the initial picture is

that of the pseudo-reaction, but while the erythema in the control arm fades within four days, it progresses in the test arm to a typical positive reaction. This indicates that the person is susceptible to diphtheria and hypersensitive to the bacillus, making immunisation necessary but likely to induce reaction.

The Schick test is not indicated in young children as they will, in any case, be susceptible and will require immunisation. But in older children and adults, Schick testing will help to identify the susceptibles.

Active immunisation

The preparations used for active immunisation against diphtheria are the following:

Toxin-Antitoxin Mixture (TAM): This was the earliest preparation used and consisted of toxin slightly underneutralised with antitoxin. This was an effective, but dangerous preparation and is no more in use. The phenol used as a preservative sometimes destroyed the antitoxin, leaving the toxin free. Another danger is that the toxicity of the toxin-antitoxin mixture is determined by the manner in which the two components are mixed. If to a given amount of antitoxin, the equivalent amount of the toxin is added all at once, the mixture is nontoxic. If instead, the same amount of toxin is added in two or more fractions, with an interval of fifteen minutes or more between the fractions, the resultant mixture will be toxic. This is known as the *Danysz phenomenon* and is a consequence of the ability of the toxins and antitoxins to combine in varying proportions. When the toxin is added in fractions, the toxin added first combines with more than its equivalent of antitoxin, leaving insufficient antitoxin behind to neutralise the toxin added subsequently.

Formol Toxoid (FT): This is a toxin that has been converted into toxoid by incubation with formaldehyde at pH 7.4–7.6 for 3–4 weeks at 37°C. Formol toxoid by itself is no more in use.

Since the adoption of mass immunisation of children, using DPT (diphtheria, pertussis, tetanus) vaccine, preparations like toxoid antitoxin mixture, toxoid antitoxin floccules, alum precipitated toxoid, purified toxoid aluminium phosphate or hydroxide, toxoid antitoxin floccules adsorbed on aluminium phosphate by themselves have all gone out of use. (Those interested in details about these may refer to the 3rd edition of this book.)

Toxoids adsorbed on to aluminium phosphate or hydroxide are combined with pertussis antigens and tetanus toxoid and are used as a combined vaccine (DPT) for immunisation. The immunisation schedules are dealt with in Chapter 68.

A preparation containing tetanus toxoid and diphtheria toxoid purified for adults should be used for adults and for those already immunised, if boosters have to be given.

Passive immunisation: This is an emergency measure, to be employed when susceptibles are exposed to infection, as when a case of diphtheria is admitted to general paediatric wards. It consists of the subcutaneous administration of 500–1000 units of antitoxin (antidiphtheritic serum, ADS). As this is a horse serum, precautions against hypersensitivity should be observed.

Combined immunisation: This consists of the administration of the first dose of toxoid on one arm, while ADS is given on the other arm, to be continued by the full course of active immunisation. Ideally, all cases that receive ADS prophylactically should receive combined immunisation. An alum-containing preparation is to be preferred for combined immunisation as the response to plain FT is not satisfactory when given with antitoxin. Purified toxoid adsorbed to aluminium phosphate (PTAP) or to aluminium hydroxide (PTAH) may be administered in two doses 0.2 ml and 0.05 ml at an interval of 1–3 months.

Standardisation of toxins and antitoxins: The

toxic activity of culture filtrates varies considerably from batch to batch. As such, their standardisation or measurement should be with reference to their biological activity. Ehrlich defined the minimum lethal dose (MLD) of diphtheria toxin as the least amount of the toxin required to kill a guinea pig weighing 250 g within 96 hours, after subcutaneous inoculation. One unit of antitoxin was defined as the smallest amount of antitoxin required to neutralise 100 MLD of toxin. Keeping a labile substance like the toxin as the standard led to inaccuracies. Toxin undergoes spontaneous denaturation into toxoid, which will combine equally well with the antitoxin. Thus, any sample of toxin will contain a variable quantity of toxoid which will vitiate standardisation of antitoxin. The antitoxin, on the other hand, is permanently stable in the freeze-dried state. Therefore, the antitoxin has been adopted as the reference preparation. Ehrlich's original antitoxin is accepted as the international standard. One antitoxin unit (AU) is defined as that amount of antitoxin that has the same total combining capacity, for toxin and toxoid together, as one unit of Ehrlich's original antitoxin.

Since toxin always contains some toxoid, two other unitages for measurement of toxin have been introduced, the Lo and L+ doses. The Lo *(Limes nul)* dose of diphtheria toxin is the largest amount of toxin that, when mixed with one unit of antitoxin and injected subcutaneously into a 250 g guinea pig, will, on the average, cause no observable reaction. As 'no reaction' is not a definite end point, in actual practice, the end point is taken as minimal local oedema. The L+ *(Limes tod)* dose of diphtheria toxin is the smallest amount of toxin that, when mixed with one unit of antitoxin and injected subcutaneously into a 250 g guinea pig, will on the average kill the animal within 96 hours. If toxin is combined with antitoxin in constant proportions, it would be expected that the difference between the L + dose and the Lo dose would be equal to 1 MLD. But when the estimations are actually made, it is found to vary from 10 to 100 MLD or more. This discrepancy is due to the presence in toxic filtrates of varying amounts of toxoid and to the ability of the toxin and antitoxin to combine in varying proportions. This is known as the *Ehrlich phenomenon.*

The use of death as an end point for titration of toxin is wasteful of animals. Romer introduced a method of titration employing the erythematous swelling produced by the intradermal injection of toxin, and its neutralisation by antitoxin. The minimum reacting dose (MRD) is the least amount of toxin that, when injected intradermally in a guinea pig, causes an erythematous flush 5 mm in diameter, visible after 36 hours. The Lf dose is the smallest amount of toxin which after mixing with one unit of antitoxin, will produce a minimal skin reaction, when injected intradermally into a guinea pig.

Ramon introduced a test tube method for titrating toxin and antitoxin based on flocculation. The flocculating or Lf unit of diphtheria toxin is that amount of toxin which flocculates most rapidly with one unit of antitoxin. The Lf unit has several advantages. It is inexpensive and rapid and does not need animals. It is also the only method available for the titration of toxoids. The amounts of toxoid in prophylactics is expressed in Lf units. Each dose of FT, PTAP and PTAH contains 25 Lf units of toxoid.

Treatment. Specific treatment of diphtheria consists of antitoxic and antibiotic therapy. Antitoxin should be given immediately when a case is suspected as diphtheria, as the fatality rate increases with delay in starting antitoxic treatment. The dosage recommended is 20,000 units intramuscularly for moderate cases and 50,000 to 100,000 units for serious cases, half the dose being given intravenously.

C. diphtheriae is sensitive to penicillin and can be cleared from the throat within a few days by penicillin treatment. Diphtheria patients are given a course of penicillin, though it only supplements and does not replace antitoxic therapy. Erythromycin is more active than penicillin in the treatment of carriers.

Other pathogenic corynebacteria

C. ulcerans is a bacillus related to *C. diphtheriae* which can cause lesions, clinically resembling diphtheria. It resembles the gravis type of the diphtheria bacillus, but it liquefies gelatin, ferments trehalose slowly and does not reduce nitrate to nitrite. It produces two types of toxins, one probably identical with the diphtheria toxin and the other resembling the toxin of *C. pseudotuberculosis*. It is pathogenic to guinea pig, the lesions produced resembling those caused by *C. diphtheriae*. It has been found to cause infection in cows, and human infection may be transmitted through cow's milk. Diphtheria antitoxin is protective.

C. acnes frequently found in acne is of doubtful pathogenicity. It is anaerobic and is now classified as Propionibacterium.

Corynebacteria of veterinary importance are the Preisz-Nocard bacillus (*C. pseudotuberculosis*), which causes pseudotuberculosis in sheep and suppurative lymphadenitis in horses; *C. pyogenes*, causing bovine mastitis and suppurative lesions in pigs; *C. renale*, causing cystitis and pyelonephritis in cattle; and *C. equi*, isolated from pneumonia in foals.

Diphtheroids

Corynebacteria resembling *C. diphtheriae* occur as normal commensals in the throat, skin, conjunctiva and other areas. These may sometimes be mistaken for diphtheria bacilli and are called diphtheroids. In general, diphtheroids stain more uniformly than diphtheria bacilli, possess few or no metachromatic granules and tend to be arranged in parallel rows (palisades) rather than cuneiform pattern. But some diphtheroids may be indistinguishable from diphtheria bacilli microscopically. Differentiation is by biochemical reactions and, more reliably, by virulence tests. The common diphtheroids are *C. pseudodiphtheriticum* (*C. hofmanni*), found in the throat and *C. xerosis*, found in the conjunctival sac.

Stain more uniformly
no metachromatic granules
arranged in parallel rows
Biochemical & Virulence T

Further Reading

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27 Bacillus

Sporogenous, rod shaped bacteria are classified into two groups, the aerobic *Bacilli* and the anaerobic *Clostridia*. The genus Bacillus consists of aerobic bacilli forming heat resistant spores They are Gram positive, but tend to be decolourised easily so as to appear Gram variable or even frankly Gram negative. They are generally motile with peritrichous flagella, the anthrax bacillus being a notable exception. Members of this genus exhibit great diversity in their properties, so that proposals have been made to subdivide them into several genera, but no acceptable scheme has been arrived at. The genus includes psychrophilic, mesophilic and thermophilic species, the maximum temperatures for vegetative growth ranging from about 25°C to above 75°C and the minimum from about −5°C to 45°C. The salt tolerance varies from less than two per cent to 25 per cent NaCl.

Their spores are ubiquitous, being found in soil, dust, water and air and constitute the commonest contaminants in bacteriological culture media. *Bacillus anthracis*, the causative agent of anthrax, is the only pathogenic species, though *B. cereus* may cause food poisoning.

Bacillus anthracis

Considerable historical interest is attached to the anthrax bacillus. It was the first pathogenic bacterium to be observed under the microscope (Pollender, 1849), the first communicable disease shown to be transmitted by inoculation of infected blood (Davaine, 1850), the first bacillus to be isolated in pure culture and shown to possess spores (Koch, 1876) and the first bacterium used for the preparation of an attenuated vaccine (Pasteur, 1881).

Morphology: The anthrax bacillus is one of the largest of pathogenic bacteria, measuring 3–10 μ × 1–1.6 μ. In tissues, *it is found singly, in pairs* or in short chains, the entire chain being surrounded by a capsule. The capsule is polypeptide in nature, being composed of a polymer of *d* (−) glutamic acid. Capsules are not formed under ordinary conditions of culture, but only if the media contain added bicarbonate or are incubated under 10–25 per cent CO_2. If grown in media containing serum, albumen, charcoal or starch, capsule formation may occur in the absence of CO_2.

In cultures, the bacilli are arranged end-to-end in long chains. The ends of the bacilli are truncated, or often concave and somewhat swollen, so that a chain of bacilli presents a 'bamboo stick' appearance. Spores are formed in culture or in the soil, but never in the animal body during life. Sporulation occurs under unfavourable conditions for growth and is encouraged by distilled water, 2% NaCl or growth in oxalated agar. Spores are formed only in the presence of oxygen. Sporulation is inhibited by anaerobic conditions and by calcium chloride. Spores are central, elliptical or oval in shape, and are of the same width as the bacillary body so that they do not cause bulging of the vegetative cell (Fig. 27.1).

The anthrax bacillus is Gram positive and nonacid fast. The spores do not stain by ordinary

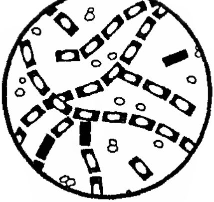

Fig. 27.1 Anthrax bacilli

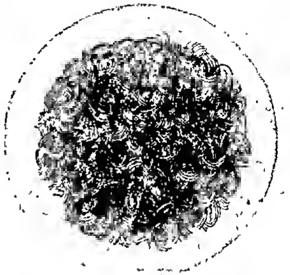

Fig. 27.2 Medusa head appearance of B. anthracis colony

methods, but can be stained differentially by special techniques. When stained with Sudan black B, fat globules may be made out within the bacilli. When blood films containing anthrax bacilli are stained with polychrome methylene blue for a few seconds and examined under the microscope, an amorphous purplish material is noticed around the bacilli. This represents the capsular material and is characteristic of the anthrax bacillus. This is called the M'Fadyean's reaction and is employed for the presumptive diagnosis of anthrax in animals.

The anthrax bacillus is nonmotile, unlike most other members of this genus.

Cultural characteristics: It is an aerobe and a facultative anaerobe, with a temperature range for growth of 12°C–45°C (optimum 35°C–37°C). The optimum temperature for sporulation is 25°C–30°C. Good growth occurs on ordinary media.

On agar plates, irregularly round colonies are formed, 2–3 mm in diameter, raised, dull, opaque, greyish white, with a frosted glass appearance. Under the low power microscope, the edge of the colony is composed of long, interlacing chains of bacilli, resembling locks of matted hair. This is called the 'Medusa head appearance' (Fig. 27.2). Virulent capsulated strains form rough colonies, while avirulent or attenuated strains form smooth colonies. On gelatin stab culture, a characteristic 'inverted fir-tree' appearance is seen, with slow liquefaction commencing from the top (Fig. 27.3). On blood agar, the colonies are nonhaemolytic, though occasional strains produce a narrow zone of haemolysis. In broth, growth occurs as floccular deposit, with little or no turbidity.

When *B. anthracis* is grown on the surface of a solid medium containing 0.05–0.5 units of penicillin/ml, in 3–6 hours the cells become large spherical and occur in chains on the surface of the agar, resembling a string of pearls. This 'string of pearls reaction' differentiates clearly *B. anthracis* from *B. cereus* and other aerobic spore bearers. Another useful test to differentiate *B. anthracis* from *B. cereus* is the former's susceptibility to gamma phage.

A selective medium (PLET medium), consisting of polymyxin, lysozyme, ethylene diamine tetra acetic acid (EDTA) and thallous acetate added to heart infusion agar, has been devised to isolate *B. anthracis* from mixtures containing other spore-bearing bacilli.

Biochemical reactions: Glucose, maltose and sucrose are fermented producing acid, but no gas. Nitrates are reduced to nitrites. Catalase is formed.

Fig 27.3 Anthrax bacillus in gelatin stab culture, showing inverted fir tree appearance

Resistance: The vegetative bacilli are not particularly resistant and are destroyed at 60°C in 30 minutes. In the carcasses of animals which had died of anthrax, the bacilli remain viable in the bone marrow for a week and in the skin for two weeks. Normal heat fixation of smears may not kill the bacilli in blood films. The spores are highly resistant to physical and chemical agents. They have been isolated from naturally infected soil after as long as 60 years. They resist dry heat at 140°C for 1–3 hours and boiling for 10 minutes. They survive in 5% phenol for weeks. $HgCl_2$ in a 1/1000 solution may fail to kill anthrax spores in less than 70 hours. Four per cent potassium permanganate kills them in 15 minutes. Destruction of the spores in animal products imported into nonendemic countries in achieved by 'duckering' in which formaldehyde is used as 2% solution at 39°C–40°C for 20 minutes for disinfection of wool and as 0.25 per cent at 60°C for six hours for animal hair and bristles. The anthrax bacillus is susceptible to sulphonamides, penicillin, erythromycin, streptomycin, tetracycline and chloramphenicol. Occasional strains resistant to penicillin have been met with.

Antigenic structure: The antigenic structure of the bacillus is not well understood. Three antigens have been characterised. The capsular polypeptide, usually found in virulent strains is a hapten. Antibody to the capsular polypeptide is not protective. The somatic polysaccharide, found as a complex in the cell wall, cross reacts serologically with the capsular polysaccharide of type 14 pneumococcus. The antibody to this antigen is not protective. The somatic protein (protective antigen) is present in the oedema fluid of anthrax lesions. It is heat labile and precipitates with its antibody in agar gel. Its antibody is protective.

Pathogenicity: In nature, anthrax is primarily a disease of cattle and sheep, and less often of horses and swine, but experimentally, most mammals are susceptible to a greater or lesser degree. Rabbits, guinea pigs and mice are susceptible, while rats and Algerian sheep are resistant. Infection can be produced with difficulty in birds. Frogs are completely resistant, while toads are very susceptible.

Following the subcutaneous inoculation of a culture into a guinea pig, the animal dies in 24–72 hours, showing a local, gelatinous, haemorrhagic oedema at the site of inoculation, extensive subcutaneous congestion and, characteristically, an enlarged, dark red, friable spleen. The blood is dark red and coagulates less firmly than normally. The bacilli are found in large numbers in the local lesion, heart blood and spleen. The bacilli are seen confined to the interior of the capillaries, where their numbers may be so great as to obstruct the flow of blood. For a long time, no true toxin could be isolated from the anthrax bacillus, and so death was considered to be due to the physical choking of capillaries by the bacteria. The pathogenesis of the disease has been clarified recently by the identification of a toxin, originally in the sterile plasma of guinea pigs

PL LH II Of Phagocytosis

dying of anthrax, and subsequently *in vitro* in media containing large amounts of serum. The toxin is a complex of three fractions that act synergistically. They have been named the oedema factor (EF or factor I), the protective antigen (PA or factor II) and the lethal factor (LF or factor III). They are not toxic individually, but the whole complex produces local oedema and generalised shock, apparently by increasing capillary permeability. Toxin production is controlled by a plasmid, the loss of which renders the strain nontoxigenic. The capsular polypeptide also acts as a virulence factor by inhibiting phagocytosis of the bacillus.

Anthrax → Cutaneous, Pulmonary, Intestinal → Fatal septicaemia

Anthrax is a zoonosis. Animals are infected by ingestion of the spores present in the soil. Direct spread from animal to animal is rare. The disease is generally a fatal septicaemia, but may sometimes be localised, resembling the cutaneous disease in man. Infected animals shed in the discharges from the mouth, nose and rectum, large numbers of bacilli, which sporulate in soil and remain as the source of infection.

Human anthrax is contracted from animals, directly or indirectly. The disease may be 1) cutaneous, 2) pulmonary, or 3) intestinal, all types leading to fatal septicaemia.

Cutaneous anthrax follows entry of the infection through the skin. The face, neck, hands, arms and back are the usual sites. The lesion starts as a papule 1–3 days after infection and becomes vesicular, containing fluid, which may be clear or bloodstained. The whole area is congested and oedematous and several satellite lesions, filled with serous or yellow fluid, are arranged round a central necrotic lesion, which is covered by a black eschar. (The name anthrax, meaning coal, comes from the black colour of the eschar.) The lesion is called a 'malignant pustule'. The disease used to be common in dock workers carrying loads of hides and skins on their bare backs and hence was known as the hide porter's disease. Cutaneous anthrax generally resolves spontaneously, but may sometimes lead to fatal septicaemia.

Pulmonary anthrax is called the wool sorter's disease because it used to be common in workers in wool factories, due to inhalation of dust from infected wool. This is a haemorrhagic pneumonia with a high fatality rate. Haemorrhagic meningitis may occur as a complication.

Intestinal anthrax is rare and occurs mainly in primitive communities who eat the carcasses of animals dying of anthrax. A violent enteritis with bloody diarrhoea occurs, with high case fatality.

Human anthrax may be industrial or non-industrial. The former is found in workers in industries such as meat packing or wool factories. Nonindustrial anthrax is often an occupational disease in those who associate frequently with animals, such as veterinarians, butchers and farmers. It may also be found in the general population. Cutaneous anthrax used to be caused by shaving brushes made with animal hair. *Stomoxys calcitrans* and other biting insects may, on occasion, transmit infection mechanically.

Anthrax is enzootic in India, the numbers of animals infected running into tens of thousands annually. The disease is rare in some countries, such as Britain, where infection is imported through contaminated hides, bone meal fertiliser and other animal products. The extent of anthrax in man is not clear, but about 10,000 cases are believed to occur annually throughout the world, mostly in rural areas. Anthrax infection in man provides permanent immunity and second attacks are extremely rare.

Laboratory diagnosis: Anthrax may be diagnosed by microscopy, culture, animal inoculation or serological demonstration of anthrax antigen in infected tissues. The immunofluorescence technique can be adopted for identifying *B. anthracis* in smears. Acute and convalescent phase sera should be obtained, since antibodies to the organism can be demonstrated by gel diffusion, complement fixation and tanned cell coated antigen agglutination techniques. The type of test to

TABLE 27.1

Differentiating features between Anlhrax and Anthracoid bacilli

Anthrax bacilli	*Anthracoid bacilli*
1 Nonmotile	Generally motile
2 Capsulated	Noncapsulated
3 Grow in long chains	Grow in short chains
4 Medusa head colony	Not present
5 No growth in penicillin agar (10 units/ml)	Grow usually
6 Haemolysis absent or weak	Usually well marked
7 Inverted fir tree growth and slow gelatin liquefaction	Rapid liquefaction
8 No turbidity in broth	Turbidity usual
9 Salicin fermentation negative	Usually positive
10 No growth at 45°C	Grows usually
11 Growth inhibited by chloral hydrate	Not inhibited
12 Susceptible to gamma phage	Not susceptible
13 Pathogenic to laboratory animals	Not pathogenic

be employed depends on the nature of the material available.

When an animal is suspected to have died of anthrax, autopsy is not permissible, as the spilt blood will lead to contamination of the soil. An ear may be cut off from the carcass and sent to the laboratory. Alternatively, swabs soaked in blood or several blood smears may be sent. The demonstration of Gram positive bacilli with the morphology of anthrax bacilli and a positive M'Fadyean's reaction will enable a presumptive diagnosis to be made. Isolation of the bacillus is easy if gross contamination has not occurred. The anthrax bacillus can often be isolated from contaminated tissues by applying them over the shaven skin of a guinea pig. It is able to penetrate though minute abrasions and produce fatal infection. If the sample received is putrid so that viable bacilli are unlikely, diagnosis may be established by Ascoli's thermoprecipitation test. The tissues are ground up in saline, boiled for five minutes, filtered and layered over the anti-anthrax serum in a narrow tube. If the tissues contain anthrax antigens, a well marked ring of precipitate will appear at the junction of the two liquids within five minutes at room temperature.

Prophylaxis: Prevention of human anthrax is mainly by general methods such as improvement of factory hygiene and proper sterilisation of animal products like hides and wool. Carcasses of animals suspected to have died of anthrax are buried deep in quicklime or cremated to prevent soil contamination.

Prevention of anthrax in animals is aided by active immunisation. The anthrax vaccine is of great historical importance. It was Pasteur's convincing demonstration of the protective effect of his anthrax vaccine in a public experiment at Pouilly-le-Fort in 1881 that marked the beginning of scientific immunoprophylaxis. Pasteur's vaccine was anthrax bacillus attenuated by growth at 42°C–43°C

As the spore is the common infective form in nature, vaccines consisting of spores of attenuated strains were developed. The Sterne vaccine

contained spores of a noncapsulated avirulent mutant strain. The Mazucchi vaccine contained spores of stable attenuated Carbazzoo strain in 2% saponin. The spore vaccines have been used extensively in animals with good results. They give protection for a year following a single injection. But they are not considered safe for human use. Alum precipitated toxoid prepared from the protective antigen has been shown to be a safe and effective vaccine for human use. It has been used in persons occupationally exposed to anthrax infection. Three doses intramuscularly at intervals of six weeks and six months induce good immunity, which can be reinforced if necessary with annual booster injections.

Treatment: Antibiotic therapy is effective in human cases, but succeeds rarely in animals as therapy is not started sufficiently early. Antibiotics have no effect on the toxin once it is formed. With penicillin and streptomycin treatment, case fatality in malignant pustule has fallen from 20 per cent to five per cent. Sclavo's serum prepared by active immunisation of asses used to be the specific treatment formerly. It may still be tried in serious cases along with antibiotics.

Anthracoid bacilli

Other species of the genus *Bacillus* are not of much medical importance. *B. subtilis* may on occasion act as an opportunistic pathogen, causing eye infections and septicaemia. *B. cereus* causes food poisoning. *B. licheniformis* has also been reported to cause food poisoning. Some species are important as producers of antibiotics such as bacitracin, tyrothricin and polymyxin. As frequent contaminants, they are often isolated in the laboratory. Most of them can be differentiated easily from the anthrax bacillus, but some, like *B. cereus* var. *mycoides* being nonmotile, may resemble *B. anthracis*. Aerobic spore bearing bacilli, having a general resemblance to *B. anthracis*, are called *anthracoid* or pseudo-anthrax bacilli. Table 27.1 lists the main differentiating features between them.

Bacillus cereus

B. cereus has recently assumed importance as a cause of food poisoning. It is widely distributed in nature and may be readily isolated from soil, vegetables and a wide variety of foods including milk, cereals, spices, meat and poultry. *B. cereus* is generally motile but nonmotile strains may occur. It resembles *B. anthracis* in many respects except that it is not capsulated and is not susceptible to gamma phage and does not react with anthrax fluorescent antibody conjugate. The animal pathogenicity test also differentiates between the two.

B. cereus produces two patterns of foodborne disease. One is associated with a wide range of foods including cooked meat and vegetables. It is characterised by diarrhoea and abdominal pain, 8–16 hours after ingestion of contaminated food. Vomiting is rare. *B. cereus* is not found in large numbers in faecal specimens from these patients. The second type is associated almost exclusively with the consumption of cooked rice, usually fried rice from Chinese restaurants. The illness is characterised by acute nausea and vomiting 1–5 hours after the meal. Diarrhoea is not common. *B. cereus* is present in large numbers in the cooked rice and faecal samples from these patients.

It has been shown that the two types of diseases are caused by strains of *B. cereus* belonging to different serotypes. The diarrhoeal disease is mostly caused by serotypes 2, 6, 8, 9, 10 or 12, while the rice associated emetic illness is caused by serotypes 1, 3, or 5. Isolates from the diarrhoeal type of disease produce an enterotoxin which causes fluid accumulation in ligated rabbit ileal loops, while strains causing the emetic type of disease produce a toxin which causes vomiting when fed to Rhesus monkeys. The emetic toxin was produced only when *B. cereus* was grown in rice but not in other media. Two mechanisms of action have been described for the enterotoxin of *B. cereus*, one involving stimulation of cAMP system and the other independent of it.

A special mannitol – egg yolk – phenol red –

(MYPA)

polymyxin agar (MYPA) medium is useful in isolating *B. cereus* from faeces and other sources.

B. cereus produces lecithinase and ferments glucose but not mannitol.

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(Mannitol; Phenol Red, Egg Yolk, Polymyxin Agar

B. cereus

2, 6, 8, 9, 10, 12 — 1, 3, 4, 5

→ Diarrhoea & abd. pain → vomiting & Nausea

→ cooked meat & vegetables → cooked Rice

→ Enterotoxin → Emetic toxin

· c̄ causes fluid accumulatⁿ in ligated ileal loop → c̄ causes vomiting when fed to Rhesus monkeys

i) ↑ cAMP ii) Independent

→ Not found in large no. in faecal specimen. → found in large no. in faecal samples & cooked Rice.

135 B. cereus — B. anthracis

→ Non capsulated. → capsulated

→ Not → Susceptible to gamma phage

→ Does not React → React c̄ anthrax flourescent Ab conjugate

MYPA

28 Clostridium

[The] genus Clostridium consists of Gram posi[ti]ve, anaerobic, spore forming bacilli. The spores [a]re wider than the bacillary bodies, giving the [b]acillus a swollen appearance, resembling a [s]pindle. Hence the name clostridium (*kloster*, [m]eaning a spindle). The genus contains bacteria [r]esponsible for three major diseases of man — [g]as gangrene, food poisoning and tetanus. Some of [th]e pathogens (e.g., *Cl. welchii* and *Cl. tetani*) are [f]ound normally in human and animal intestines. [M]any species are pathogenic, but most are sapro[p]hytes found in soil, water and decomposing [p]lant and animal matter. Some (e.g., *Cl. aceto[b]utylicum*) are of industrial importance, used for [th]e production of chemicals such as acetone and [b]utanol.

Clostridia are highly pleomorphic. They are [r]od shaped, usually 3–8 μ x 0.4–1.2μ in size. [L]ong filaments and involution forms are common. [S]pore formation occurs with varying frequency in [d]ifferent species. Some (*Cl. sporogenes*) sporu[l]ate readily, while others (*Cl. welchii*) do so incon[s]tantly. Sporulation takes place in the animal [b]ody also. The shape and position of spores vary [i]n different species and these are of use in the [i]dentification and classification of clostridia. [S]pores may be 1) central or equatorial, giving the [b]acillus a spindle shape (*Cl. bifermentans*), [2]) subterminal, the bacillus appearing club shaped [(]*Cl. welchii*), 3) oval and terminal, resembling a [t]ennis racket (*Cl. tertium*), or 4) spherical and [t]erminal, giving a drumstick appearance (*Cl. [t]etani*).

Clostridia are motile with peritrichate flagella, with few exceptions such as *Cl. welchii*, and *Cl. tetani* type VI, which are nonmotile. The motility is slow and has been described as 'stately'. *Cl. welchii* and *Cl. butyricum* are capsulated, while others are not.

Clostridia are easily stained. They are Gram positive, but in older cultures, cells are often Gram variable, or even frankly Gram negative.

Clostridia are anaerobic. The sensitivity to oxygen varies in different species. Some (e.g., *Cl. oedematiens*) are exacting anaerobes and die on exposure to oxygen, while some others (e.g., *Cl. histolyticum*) are aerotolerant and may even grow aerobically. More important than the absence of oxygen is the provision of a sufficiently low redox potential (Eh) in the medium. This can be achieved by adding reducing substances such as unsaturated fatty acids, ascorbic acid, glutathione, cysteine, thioglycollic acid, alkaline glucose, sulphites or metallic iron. A small concentration of CO_2 appears to enhance growth. The optimum temperature for pathogenic clostridia is 37°C. Some saprophytic clostridia are thermophilic and others psychrophilic. The optimum pH is 7–7.4.

Growth is relatively slow on solid media. Colonial characteristics are variable. Some species are haemolytic on blood agar. A very useful medium is Robertson's cooked meat broth. It contains unsaturated fatty acids which take up oxygen, the reaction being catalysed by haematin in the meat, and also sulphydryl compounds which bring about a reduced OR potential. Clostridia grow in the medium, rendering the broth turbid. Most species produce gas. Saccharolytic

species turn the meat pink. Proteolytic species turn the meat black and produce foul and pervasive odours. In litmus milk medium, the production of acid, clot and gas can be detected.

The vegetative cells of clostridia do not differ from nonsporing bacilli in their resistance to physical and chemical agents. The spores exhibit a pronounced, but variable resistance to heat, drying and disinfectants. Spores of *Cl. botulinum* survive boiling for 3–4 hours and, even at 105°C, are not killed completely in less than 100 minutes. Spores of most strains of *Cl. welchii* are destroyed by boiling for less than five minutes, but spores of some Type A strains that cause food poisoning survive for several hours. *Cl. tetani* spores persist for years in dried earth. Spores of some strains of *Cl. tetani* resist boiling for 15–90 minutes, though in most cases, they are destroyed within five minutes. All spores are killed by autoclaving at 121°C within 10 minutes. Spores are particularly resistant to phenolic disinfectants. Formaldehyde is not very active and spores may sometimes survive immersion in 2% solution for upto five days. Halogens are effective and 1% aqueous iodine solution kills spores within three hours. Glutaraldehyde (2% at pH 7.5–8.5) is very effective in killing spores. Clostridia are sensitive to antibiotics. Of the antibiotics used commonly, tetracyclines are the most active, penicillin less so and chloramphenicol still less.

Clostridia can produce disease only when the conditions are appropriate. Their invasive powers are limited. Pathogenic clostridia form powerful exotoxins. *Cl. botulinum* is totally noninvasive and practically noninfectious. Botulism is due to ingestion of preformed toxin in food. *Cl. tetani* has slight invasive properties and is confined to the primary site of lodgement. Tetanus results from the action of the potent exotoxin it produces. The gas gangrene clostridia, besides being toxigenic, are also invasive and can spread along tissues and even cause septicaemia.

Many methods have been adopted for the classification of clostridia. These include morphological features such as the shape and position of spores and biochemical features such as saccharolytic and proteolytic capacities (Table 28.1). Clostridia of medical importance may also be considered under the diseases they produce (see classification below).

A. The gas gangrene group:

1. Established pathogens	*Cl. welchii*, *Cl. septicum*, *Cl. oedematiens*
2. Less pathogenic	*Cl. histolyticum*, *Cl. fallax*
3. Doubtful pathogens	*Cl. bifermentans*, *Cl. sporogenes*

B. Tetanus — *Cl. tetani*

C. Food poisoning:

1. Gastroenteritis	*Cl. welchii* (Type A)
2. Botulism	*Cl. botulinum*

D. Acute colitis — *Cl. difficile*.

CLOSTRIDIUM WELCHII

(Cl. perfringens, Bacillus aerogenes capsulatus; B. phlegmonis emphysematosae.)

This bacillus was originally cultivated by Achalme (1891) but was first described in detail by Welch and Nuttall (1892), who isolated it from the blood and organs of a cadaver. This is the most important of the clostridia causing gas gangrene. It also produces food poisoning and necrotic enteritis in man and many serious diseases in animals.

Cl. welchii is a normal inhabitant of the large intestine of man and animals. It is found in faeces and contaminates the skin of the perineum, buttocks and thighs. The spores are commonly found in soil, dust and air.

Morphology: It is a plump, Gram positive bacillus with straight, parallel sides and rounded or truncated ends, about 4–6μ x 1μ, usually occurring singly or in chains or small bundles. It is pleomorphic, and filaments and involution forms are common. It is capsulated and nonmotile. Spores are central or subterminal, but are rarely seen in artificial culture or in material from pathological lesions, and their absence is one of

the characteristic morphological features of *Cl welchii.*

Cultural characteristics: It is an anaerobe, but can also grow under micro-aerophilic conditions. Oxygen is not actively toxic to the bacillus and cultures do not die on exposure to air, as happens with some fastidious anaerobes. It grows over a pH range 5.5–8.0 and temperature range of 20°–50°C. Though usually grown at 37°C, a temperature of 45°C is optimal for many strains. The generation time at this temperature may be as short as ten minutes. This property can be utilised for obtaining pure cultures of *Cl. welchii*. Robertson's cooked meat broth inoculated with mixtures of *Cl. welchii* and other bacteria and incubated at 45°C for 4–6 hours serves as an enrichment. Subcultures from this onto blood agar plates yield pure or predominant growth of *Cl. welchii*.

Good growth occurs in Robertson's cooked meat medium. The meat is turned pink, but is not digested. The culture has an acid reaction and a sour odour.

In litmus milk, fermentation of lactose leads to formation of acid, which is indicated by the change in the colour of litmus from blue to red. The acid coagulates the casein (acid clot) and the clotted milk is disrupted due to the vigorous gas production. The paraffin plug is pushed up and shreds of clot are seen sticking to the sides of the tube. This is known as 'stormy fermentation'.

After overnight incubation on rabbit, sheep or human blood agar, colonies of most strains show a 'target haemolysis', resulting from a narrow zone of complete haemolysis due to theta toxin and a much wider zone of incomplete haemolysis due to alpha toxin. This double zone pattern of haemolysis may fade on longer incubation.

Biochemical reactions: Glucose, maltose, lactose and sucrose are fermented with the production of acid and gas. It is indole negative, MR positive and VP negative. H_2S is formed abundantly. Most strains reduce nitrates.

Resistance: Spores are usually destroyed within five minutes, by boiling but those of the 'food-poisoning' strains of Type A and certain Type C strains resist boiling for 1–3 hours. Autoclaving at 121°C for 15 minutes is lethal. Spores are resistant to the antiseptics and disinfectants in common use.

Classification: *Cl. welchii* strains are classified into five types, A to E, based on the toxins they produce. Though the bacillus produces a large number of toxins, typing depends on the four 'major toxins'. Typing is done by neutralisation tests with specific antitoxins by intracutaneous injection in guinea pigs or intravenous injection in mice.

Toxins: *Cl. welchii* is one of the most prolific of toxin producing bacteria, forming at least 12 distinct toxins, besides many other enzymes and biologically active soluble substances. The four 'major toxins', alpha, beta, epsilon and iota, are predominantly responsible for pathogenicity.

Alpha (α) toxin is produced by all types of *Cl. welchii* and most abundantly by Type A strains. This is the most important toxin biologically and is responsible for the profound toxaemia of gas gangrene. It is lethal, dermonecrotic and haemolytic. It is a phospholipidase (lecithinase C) which, in the presence of Ca^{++} and Mg^{++} ions, splits lecithin into phosphoryl choline and a diglyceride. This reaction is seen as an opalescence in serum or egg yolk media and is specifically neutralised by the antitoxin. When *Cl. welchii* is grown on a medium containing 6% agar, 5% Fildes' peptic digest of sheep blood and 20% human serum, with the antitoxin spread on one half of the plate, colonies on the half without the antitoxin will be surrounded by a zone of opacity. There will be no opacity around the colonies on the half of the plate with the antitoxin, due to the specific neutralisation of the alpha toxin. This specific lecithinase effect, known as the Nagler reaction, is a useful test for the rapid detection of *Cl. welchii* in clinical specimens (Fig. 28.1). The incorporation of neomycin sulphate in

TABLE 28.1

A morphological and biochemical classification of Clostridia

Position of spores	Both proteolytic and saccharolytic		Slightly proteolytic but not saccharolytic	Saccharolytic but not proteolytic	Neither proteolytic nor saccharolytic
	Proteolytic predominating	Saccharolytic predominating			
Central or subterminal	*Cl. bifermentans* *Cl. botulinum* A,B,F. *Cl. histolyticum* *Cl. sordelli* *Cl. sporogenes*	*Cl. welchii* *Cl. septicum* *Cl. chauvoei* *Cl. oedematiens*	.. *Cl. difficile*	*Cl fallax* *Cl. botulinum* C,D,E	...
Oval and terminal	...	...		*Cl. tertium*	*Cl. cochlearum*
Spherical and terminal	...		*Cl tetani*	*Cl. tetanomorphum* *Cl. sphenoides*	...

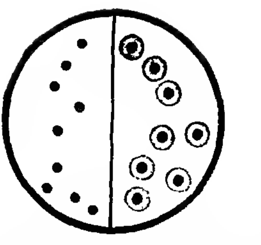

Fig 28.1 Nagler reaction *Cl welchii* colonies on the right half of the plate are surrounded by haloes, while colonies on the left half (containing antiserum to alpha toxin) have no haloes around them

the medium makes it more selective, inhibiting coliforms and aerobic spore bearers. Human serum may be replaced by 5% egg yolk. The opalescence in the egg yolk media may be produced by other lecithinase forming bacteria also (*Cl. oedematiens, Cl bifermentans*, some vibrios, some aerobic spore formers). In the case of these bacteria, the reaction is not neutralised by *Cl. welchii* antitoxin, except with *Cl. bifermentans*, which produces a serologically related lecithinase.

The alpha toxin is haemolytic for the red cells of most species, except horse and goat, due to its action on the phospholipids on the erythrocyte membranes. The lysis is of the hot-cold variety, being best seen after incubation at 37°C followed by chilling at 4°C. It is relatively heat stable and is only partially inactivated by boiling for five minutes.

Beta (β), *epsilon* (ε) and *iota* (ι) toxins have lethal and necrotising properties. *Gamma* (γ) and *eta* (η) toxins have minor lethal actions. *Delta* (δ) toxin has lethal effect and is haemolytic for the red cells of even-toed ungulates (sheep, goats, pigs, cattle). *Theta* (θ) toxin is an oxygen labile haemolysin antigenically related to streptolysin O. It is also lethal and a general cytolytic toxin. *Kappa* (κ) toxin is a collagenase. *Lambda* (λ) toxin is a proteinase and gelatinase. *Mu* (μ) toxin is a hyaluronidase and *Nu* (ν) toxin a deoxyribonuclease.

Besides the toxins, *Cl. welchii* also produces other soluble substances, some of which possess enzymatic properties. These include the enzymes which destroy the blood group substances A and H, a neuraminidase which destroys myxovirus receptors on red blood cells, a substance which renders red blood cells panagglutinable by exposing their T antigens, a haemagglutinin active against red blood cells of man and most animals, a fibrinolysin, a haemolysin distinct from alpha, theta and delta toxins, histamine, a 'bursting factor' which has a specific action on muscle tissue and may be responsible for the characteristic muscle lesions in gas gangrene, and a 'circulating factor' which can cause an increase in the adrenaline sensitivity of the capillary bed and also inhibit phagocytosis.

Pathogenicity: Cl. welchii produces the following human infections:

Gas gangrene: *Cl. welchii* Type A is the predominant agent causing gas gangrene. It may occur as the sole aetiological agent, but is more commonly seen in association with other clostridia as well as nonclostridial anaerobes and even aerobes. All clostridial wound infections do not result in gas gangrene. More commonly, they lead only to 'wound contamination', or, anaerobic cellulitis. It is only when muscle tissues are invaded that gas gangrene (anaerobic myositis) results.

Food poisoning: Some strains of Type A (food-poisoning strains) can produce food poisoning. They are characterised by the marked heat resistance of their spores and the feeble production of alpha and theta toxins. They have been shown to produce a heat labile enterotoxin, which, like the enterotoxins of *V. cholerae* and enteropathogenic *E. coli*, leads to fluid accumulation in the rabbit ileal loop.

Food poisoning by *Cl. welchii* is usually caused by a cold or warmed up meat dish. After an incu-

TABLE 28 2

Toxins produced by *Cl. welchii* types

Type pathogenicity	Major Toxins				Minor Toxins							
	α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν
A Gas gangrene: wound infections; septicaemia	+++	−	−	−	−	−	±	±	±	−	±	±
Food poisoning	+++	−	−	−	−	−	−	±	±	−	±	++
B Lamb dysentery	+++	+++	++	−	−+	±	−	++	−	+++	+++	++
C Enteritis in animals	+++	+++	−	−	−	−	−	+++	+++	−	−	±
Enteritis necroticans in man	+++	++	−	−	++	−	−	−	−	−	−	+++
D Enterotoxaemia of sheep	+++	−	+++	−	−	−	−	++	++	++	±	±
E Doubtful pathogen of sheep and cattle	+++	−	−	+++	−	−	−	++	++	++	±	±

bation period of 8–24 hours, abdominal pain, diarrhoea and vomiting set in. The illness is self-limited, and recovery occurs in 24-48 hours. Diagnosis is made by isolating heat resistant *Cl. welchii* Type A from the faeces and food. As this may be present sometimes in normal intestine, isolation from faeces, except in large numbers, is not meaningful. Isolation from food has to be attempted by direct plating on selective media, as the bacillus is present in food mainly as the vegetative cells

Gangrenous appendicitis: *Cl welchii* Type A (and occasionally Type D) strains have been isolated from gangrenous appendicitis. The demonstration of antitoxin in these patients and the beneficial effects of the administration of antitoxin also suggest the aetiological role of the bacillus in this condition. It has been proposed that the toxaemia and shock in some cases of intestinal obstruction and peritonitis may be due to the toxins of *Cl. welchii*

Necrotising enteritis: A severe and often fatal necrotising jejunitis (enteritis necroticans, pig-bel) has been found to be caused by Type C strains. This condition is rare, but sporadic cases and outbreaks have been reported from different countries — Germany, New Guinea, East Africa, Thailand and Nepal. Active immunisation with *Cl. welchii* Type C toxoid has been shown to give protection against this condition.

Necrotising colitis This is a very rare but fatal disease in which large segments of the colon become gangrenous. This has been proposed, but not proved to be due to *Cl. welchii* infection.

Biliary tract infection: *Cl. welchii* has been reported to produce two rare but serious infections of the biliary tract — acute emphysematous cholecystitis and post-cholecystectomy septicaemia.

Endogenous gas gangrene of intra-abdominal origin: Gas gangrene of the abdominal wall has been reported as an infrequent complication of abdominal surgery. The infection is endogenous, the organism being derived from the gut and contaminating the abdominal wall during surgery. Gas gangrene of the thigh as a result of infection tracking from the abdomen has also been reported.

Brain abscess and meningitis: Brain abscess and meningitis due to *Cl. welchii* have been reported very rarely.

Panophthalmitis: Panophthalmitis due to *Cl. welchii* has occasionally followed penetrating eye injuries.

Thoracic infections: Clostridial infection of the chest cavity may follow penetrating wounds of the thorax. This is more often seen in battle casualties than in civilian situations.

Urogenital infections: Infection of the urinary tract may occasionally follow surgical procedures such as nephrectomy. Clostridial infection of the uterus is a serious and not infrequent condition, commonly associated with septic abortion. Septicaemia is common in this condition.

CLOSTRIDIUM SEPTICUM

This bacillus was first described by Pasteur and Joubert (1887) and called *Vibrion septique*. This is a pleomorphic bacillus, about 3–8μ x 0.6μ in size, forming oval, central or subterminal spores. It is motile by peritrichate flagella. Growth occurs anaerobically on ordinary media. The colonies are irregular and transparent initially, turning opaque on continued incubation. Haemolysis occurs on horse blood agar. Growth is promoted by glucose. It is saccharolytic and produces abundant gas.

Six groups have been recognised, based on somatic and flagellar antigens. *Cl. septicum* produces at lease four distinct toxins and fibrinolysin. The alpha toxin is haemolytic, dermonecrotic and lethal, the beta toxin is a leucotoxic deoxyribonuclease, the gamma toxin a hyaluronidase and the delta toxin an oxygen labile haemolysin.

Cl. septicum is found in the soil or animal intestine. It is associated with gas gangrene in man, usually with other clostridia. It also causes 'braxy' in sheep and 'malignant oedema' in cattle and sheep. *Cl. septicum* is so closely related to *Cl. chauvoei* that the two are grouped as a single species. *Cl. septicum* can escape from the intes-

tines of man and animals and invade the tissues shortly after death. Therefore, the presence of *Cl. septicum* in pathological specimens must be interpreted with caution.

CLOSTRIDIUM OEDEMATIENS
(Cl. novyi)

This is a large, stout, pleomorphic, Gram positive bacillus about 4–10μ x 1–2μ in size, with large, oval, subterminal spores. It is widely distributed in soil. It is a strict anaerobe, readily inactivated by the exposure of cultures to air. Four types (A to D) are recognised, based on the production of toxins. Only Type A is of medical importance, as it causes gas gangrene. Gas gangrene caused by *Cl. oedematiens* is characterised by high mortality and large amounts of oedema fluid with little or no observable gas in infected tissue. Other types produce veterinary diseases. *Cl. oedematiens* is a potent toxin producer. The following toxins have been identified: *alpha* — necrotising and lethal; *beta* — haemolytic, necrotising, lethal, lecithinase; *gamma* — haemolytic, necrotising, lecithinase, *delta* — oxygen labile haemolysin; *epsilon* — lipase, opalescence in egg yolk; *zeta* — haemolysin; *eta* — tropomyosinase; *theta* — opalescence in egg yolk.

CLOSTRIDIUM HISTOLYTICUM

This is an actively proteolytic clostridium, forming oval, subterminal, bulging spores. It is aerotolerant and some growth may occur even in aerobic cultures. It forms at least five distinct toxins: *alpha* — lethal, necrotising; *beta* — collagenase, gelatinase; *gamma* — proteinase, gelatinase; *delta* — elastinase, gelatinase; *epsilon* — oxygen labile haemolysin. Spores are found in soil, but the distribution seems to be sparse and uneven. It is associated with gas gangrene in man, but less often than other clostridia. It is of interest that Dhayagude and Purandare (1949) found *Cl. histolyticum* to be the sole pathogen in four out of 25 cases of gas gangrene from Bombay.

GAS GANGRENE

Oakley (1954) has defined gas gangrene as 'a rapidly spreading, oedematous myonecrosis, occurring characteristically in association with severe wounds of extensive muscle masses that have been contaminated with pathogenic clostridia, particularly with *Cl. welchii*'. The disease had been referred to in the past as 'malignant oedema'. Other descriptive terms that have been used are 'anaerobic (clostridial) myositis' and 'clostridial myonecrosis'. Gas gangrene appears to have been described by Hippocrates (460–355 BC), but the disease did not come into prominence until World War I. In 1914, the incidence of gas gangrene among the wounded in the British Expeditionary Force was as high as 12 per cent with a case fatality of upto 25 per cent. With improvements in the management of war casualties, the incidence fell to one per cent by 1918.

Gas gangrene is characteristically a disease of war, in which extensive wounds with heavy contamination are so common. In civilian life, the disease generally follows road accidents or other types of injury involving crushing of large muscle masses. Rarely, it may even follow surgical operations.

The bacteriology of gas gangrene is varied. Rarely, it is due to infection with a single clostridium. Generally, several species of clostridia are found in association with anaerobic streptococci and facultative anaerobes such as *E. coli*, proteus and staphylococci. Amongst the pathogenic clostridia, *Cl. welchii* is the most frequently encountered (approximately 60 per cent), *Cl. oedematiens* and *Cl. septicum* being the next common (20–40 per cent), and *Cl. histolyticum* less often. Other clostridia usually found are *Cl. sporogenes*, *Cl. fallax*, *Cl. bifermentans*, *Cl. sordellii*, *Cl. aerofoetidum* and *Cl. tertium*. These may not be pathogenic by themselves. The relative incidence of the different species varies in different series of cases and may be a reflection of the distribution of the species in different soils.

Clostridia enter the wounds usually along with implanted foreign particles such as soil (particu-

larly, manured or cultivated soil), road dust, bits of clothing or shrapnel. Clostridia may also be present on the normal skin, especially on the perineum and thighs. Infection may at times be endogenous. Gas gangrene may occasionally follow clean surgical procedures (especially amputations for vascular disease) and even injections (especially of adrenaline).

The mere presence of clostridia in wounds does not constitute gas gangrene. MacLennan has distinguished three types of anaerobic wound infections:

1. Simple wound contamination with no invasion of the underlying tissue, resulting in little more than some delay in wound healing.

2. Anaerobic cellulitis in which clostridia invade the fascial planes, with minimal toxin production and no invasion of muscle tissues. The disease is gradual in onset and may vary from a limited 'gas abscess' to extensive involvement of a limb. The infecting clostridia are typically of low invasive power and poor toxigenicity. There is a seropurulent discharge from the wound with an offensive odour. Toxaemia is absent or negligible and the prognosis good. Anaerobic cellulitis is two or three times more frequent than gas gangrene amongst battle casualties, but is rare in civilian life.

3. Anaerobic myositis or gas gangrene, which is the most serious, associated with clostridial invasion of healthy muscle tissues and abundant formation of exotoxins. Gas gangrene results only if, in the wound, the conditions favourable for clostridial multiplication exist. The most important of these is a low oxygen tension. This is achieved ideally in battle wounds in which there are implanted bullets or shell fragments, along with bits of clothing and soil particles. The ionised calcium salts and silicic acid in the soil cause tissue necrosis. Crushing or tearing of the arteries produces anoxia of the muscle. Extravasation of blood increases the pressure on the capillaries, reducing the blood supply still further. The Eh and pH of the damaged tissues fall, and these changes along with the chemical changes that occur within the damaged and anoxic muscles, including breakdown of carbohydrates and liberation of aminoacids from proteins, provide an ideal pabulum for the proliferation of anaerobes. Extravasated haemoglobin and myohaemoglobin are reduced and cease to act as oxygen carriers. As a result, aerobic oxidation is halted and anaerobic reduction of pyruvate to lactate leads to a further fall in Eh.

The clostridia multiply and elaborate toxins which cause further tissue damage. Though the exact details are not known, it is possible to surmise the role of the various toxins in the development of the disease. The lecithinases damage cell membranes and increase capillary permeability, leading to extravasation and increased tension in the affected muscles, causing further anoxic damage. The haemolytic anaemia and haemoglobinuria seen in *Cl. welchii* infections are due to the lysis of erythrocytes by the alpha toxin. The collagenases destroy collagen barriers in the tissues and hyaluronidases breakdown the intercellular substances, furthering invasive spread by the clostridia. The abundant production of gas reduces the blood supply still further by pressure effects, extending the area of anoxic damage. It becomes, thus, possible for the infection to spread from the original site, making the lesion a progressive one.

The incubation period may be as short as seven hours or as long as six weeks after wounding, the average being 10–48 hours with *Cl. welchii*, 2–3 days with *Cl. septicum* and 5–6 days with *Cl. oedematiens* infection. The disease develops with increasing pain, tenderness, and oedema of the affected part, along with systemic signs of toxaemia. There is a thin watery discharge from the wound, which later becomes profuse and serosanguineous. Accumulation of gas makes the tissues crepitant. In untreated cases, the disease process extends rapidly and inexorably. Profound toxaemia and prostration develop and death occurs due to circulatory failure.

Laboratory diagnosis: The diagnosis of gas gangrene must be made primarily on clinical grounds, and the function of the laboratory is

only to provide confirmation of the clinical diagnosis as well as identification and enumeration of the infecting organisms. Bacteriological examination also helps to differentiate gas gangrene from anaerobic streptococcal myositis, which may be indistinguishable from it clinically in the early stages. In the latter, Gram stained films show large numbers of streptococci and pus cells, but not bacilli, contrasting with the scanty pus cells and diverse bacterial flora seen in films from gas gangrene.

The specimens to be collected are 1) films from the muscles at the edge of the affected area, from the tissue in the necrotic area and from the exudate in the deeper parts of the wound; 2) exudates from the parts where infection appears to be most active and from the depths of the wound, to be collected with a capillary pipette or a swab; and 3) necrotic tissue and muscle fragments

Gram stained films give presumptive information about the species of clostridia present and their relative numbers. The presence of large numbers of regularly shaped Gram positive bacilli without spores is strongly suggestive of *Cl. welchii* infection. 'Citron bodies' and boat or leaf shaped pleomorphic bacilli with irregular staining suggest *Cl septicum.* Large bacilli with oval, subterminal spores indicate *Cl. oedematiens.* Slender bacilli, with round, terminal spores may be *Cl. tetani* or *Cl. tetanomorphum.*

Aerobic and anaerobic cultures are made on fresh horse blood agar and heated blood agar. It is advisable to use 5–6 per cent agar in the plates to prevent swarming. A plate of serum or egg yolk agar, with *Cl. welchii* antitoxin spread on one half is used for 'Nagler reaction'. Four tubes of Robertson's cooked meat broth are inoculated and heated at 100 °C for 5,10,15 and 20 minutes, incubated and subcultured on blood agar plates after 24–48 hours, to differentiate the organisms with heat resistant spores. Blood cultures are often positive, especially in *Cl. welchii* and *Cl. septicum* infections, though it should be remembered that *Cl. welchii* bacteraemia may occur without gas gangrene. The isolates are identified based on their morphological, cultural, biochemical and toxigenic characters.

Prophylaxis and therapy: Surgery is the most important prophylactic and therapeutic measure in gas gangrene. All damaged tissues should be removed promptly and the wounds irrigated to remove blood clots, necrotic tissue and foreign materials. In established gas gangrene, uncompromising excision of all affected parts may be life saving. Where facilities exist, hyperbaric oxygen may be beneficial in treatment.

Chemotherapy and antibodies are of value in *prophylaxis, in combination with adequate* surgery. Gas gangrene clostridia are susceptible to sulphonamide, metronidazole, penicillin and tetracycline, but not to chloramphenicol and aminoglycosides. Antibacterial therapy appears to be of little benefit in the treatment of established gas gangrene.

Passive immunisation with polyvalent antitoxin (anti-gas gangrene serum) is generally advocated in the prophylaxis of gas gangrene in cases with extensive soiled wounds. The dose recommended is 10,000 units of *Cl. welchii* antitoxin, 10,000 units of *Cl oedematiens* antitoxin and 5,000 units of *Cl. septicum* antitoxin, given intramuscularly or, in emergencies, intravenously. Precaution should be taken to guard against hypersensitivity to horse serum. Antitoxins are used therapeutically also, at least thrice, the prophylactic dose being given intravenously and repeated every six hours. if necessary.

Active immunisation with toxoids has been found, *experimentally, to induce good antitoxic* response, but the utility of this method in prophylaxis is uncertain.

CLOSTRIDIUM TETANI

Cl. tetani is the causative organism of tetanus. Tetanus has been known from very early times, having been described by Hippocrates and Aretaeus. Carle and Rattone (1884) transmitted the disease to rabbits. Nicolaier (1884), studying the

experimental disease, suggested that the manifestations of tetanus were due to a strychnine-like poison produced by the bacillus multiplying locally. Rosenbach (1886) demonstrated a slender bacillus with round terminal spores in a case of tetanus. The final proof of the aetiological role of the bacillus in tetanus was furnished by Kitasato (1889) who isolated it in pure culture and reproduced the disease in animals by inoculation of pure cultures.

Cl. tetani is widely distributed in soil and in the intestine of man and animals. It is ubiquitous and has been recovered from a wide variety of other sources, including street and hospital dust, cotton wool, plaster of paris, bandages, catgut, talc, wall plaster, clothing, etc. It may occur as an apparently harmless contaminant in wounds.

Fig 28.2 *Cl. tetani*, some with spores and some without

Morphology

It is a Gram positive, slender bacillus, about 4–8μ x 0.5μ, though there may be considerable variation in length. It has a straight axis, parallel sides and rounded ends. It occurs singly and occasionally in chains. The spores are spherical, terminal and bulging, giving the bacillus the characteristic 'drumstick' appearance (Fig. 28.2). The morphology of the spore depends on its stage of development and the young spore may be oval rather than spherical. It is noncapsulated and motile by peritrichate flagella. Young cultures are strongly Gram positive, but older cells show variable staining and may even be Gram negative.

Cultural characteristics

It is an obligatory anaerobe that grows only in the absense of oxygen. The optimum temperature is 37°C and pH 7.4. It grows on ordinary media. Growth is improved by blood and serum, but not by glucose. Surface colonies are difficult to obtain as the growth has a marked tendency to swarm over the surface of the agar, especially if the medium is moist. An extremely fine, translucent film of growth is produced that is practically invisible, except at the delicately filamentous advancing edge. This property enables the separation of *Cl. tetani* from mixed cultures. If the water of condensation at the bottom of a slope of nutrient agar is inoculated with the mixed culture, after incubation anaerobically for 24 hours, subcultures from the top of the tube will yield a pure growth of the tetanus bacillus (Fildes' technique).

In deep agar shake cultures, the colonies are spherical fluffy balls, 1–3 mm in diameter, made up of filaments with a radial arrangement. In gelatin stab cultures a fir tree type of growth occurs, with slow liquefaction.

It grows well in Robertson's cooked meat broth, with turbidity and some gas formation. The meat is not digested, but is turned black on prolonged incubation.

On horse blood agar, α-haemolysis is produced, which later develops into β-haemolysis, due to the production of a haemolysin (tetanolysin).

Biochemical reactions

Cl. tetani has feeble proteolytic, but no saccharolytic property. It does not attack any sugar.

It forms indole. It is MR and VP negative. H_2S is not formed. Nitrates are not reduced. Gelatin liquefaction occurs very slowly. A greenish fluorescence is produced on media containing neutral red (as on MacConkey's medium).

Resistance

The resistance of tetanus spores to heat appears to be subject to strain differences. Most are killed by boiling for 10–15 minutes, but some resist boiling for upto three hours. When destruction of spores is to be ensured, autoclaving at 121°C for 20 minutes is recommended. On the other hand, when heat is applied in order to free cultures of *Cl. tetani* from nonsporing contaminants, it is important not to exceed 80°C for 10 minutes, as even this mild treatment can cause considerable destruction. Spores are able to survive in soil for years. Spores are resistant to most antiseptics. They are not destroyed by 5% phenol or 0.1% mercuric chloride solution in two weeks or more. Iodine (1% aqueous solution) and hydrogen peroxide (10 volumes) kill the spores within a few hours.

Classification

Ten serological types have been recognised based on agglutination (types I to X). Type VI contains nonflagellated strains. All other types possess type specific flagellar antigens. All the types produce the same toxin, which is neutralised by antitoxin produced against any one type.

Toxins: Cl. tetani produces at least two distinct toxins — a haemolysin *(tetanolysin)* and a powerful neurotoxin *(tetanospasmin)*. The two are antigenically and pharmacologically distinct and their production is mutually independent. A third toxin, a nonspasmogenic, peripherally active neurotoxin has been identified recently. It is not known whether this plays any role in the pathogenesis of tetanus.

Tetanolysin is a heat labile, oxygen labile haemolysin, antigenically related to the oxygen labile haemolysins produced by *Cl. welchii, Cl. oedematiens* and *Str. pyogenes.* This lysin is active against erythrocytes of several animal species, especially the rabbit and the horse. Its pathogenic role is unknown. It may act as a leucotoxin.

Tetanospasmin is the toxin responsible for the clinical manifestation of tetanus. It is oxygen stable, but relatively heat labile, being inactivated at 65°C in five minutes. It gets toxoided spontaneously or in the presence of low concentrations of formaldehyde. It is a good antigen and is specifically neutralised by the antitoxin. The toxin has been crystallised. The toxin constitutes 5–10 per cent of the bacterial weight. It exists in two forms. A monomer of MW 68,000 which is toxic and a dimer which is nontoxic but antigenic.

It appears to be a simple protein. The purified toxin is active in extremely small amounts and has an MLD for mice of about $50–75 \times 10^{-6}$ mg. The amount of toxin produced depends on the strain of bacillus and the type of culture medium used. There is considerable variation in the susceptibility of different species of animals to the toxin The horse is the most susceptible. Guinea pigs, mice, goats and rabbits are susceptible in that descending order. Birds and reptiles are highly resistant. Frogs which are normally insusceptible, may be rendered susceptible by elevating their body temperature.

Pathogenicity: Cl. tetani has little invasive power. The spore implanted in a wound can germinate and multiply only if the conditions are favourable. The toxin produced locally is absorbed by the motor nerve endings and transported to the central nervous system along the axis cylinders of the peripheral nerve. The toxin is specifically and avidly fixed by gangliosides of the grey matter of the nervous tissue. Tetanospasmin resembles strychnine in its effects. The tetanus toxin specifically blocks synaptic inhibition in the spinal cord, presumably at inhibitory terminals that use glycine as neurotransmitter. The toxin acts presynaptically, unlike strychnine which acts postsynaptically. The abolition of spinal inhibition causes uncontrolled spread of impulses initiated

anywhere in the central nervous system. This results in muscle rigidity and spasms due to the simultaneous contraction of agonists and antagonists, in the absence of reciprocal inhibition. The convulsion pattern is determined by the most powerful muscles at a given joint, and in most animals is characterised by tonic extension of the body and of all limbs.

The toxicity of tetanospasmin is influenced by the route by which it is administered. Given orally, it is destroyed by the digestive enzymes and is without effect. Subcutaneous, intramuscular and intravenous injections are equally effective. Intraneural injections are more lethal and injections directly into the central nervous system very much more so. The route of administration also modifies the clinical picture. Experimental tetanus may, accordingly, be of the 'local', 'ascending' or 'descending' variety. These differences are related to the manner in which the toxin reaches and is disseminated in the central nervous system.

When the toxin is inoculated intramuscularly in one of the hindlimbs, tonic spasms of the muscles of the inoculated limb appear first. This is known as local tetanus and is due to the toxin acting on the segment of the spinal cord containing the motor neurons of the nerves supplying the inoculated area. Subsequent spread of the toxin up the spinal cord causes 'ascending tetanus'. The opposite hindlimb, trunk and forelimbs are involved in an ascending fashion. If the toxin is injected intravenously, spasticity develops first in the muscles of the head and neck and then spreads downwards — 'descending tetanus'. This type resembles the naturally occurring tetanus in man.

TETANUS

Tetanus results from the infection of a wound or raw surface with the tetanus bacillus or, more rarely, from the parenteral injection of contaminated substances and is characterised by tonic muscular spasms, usually commencing at the site of infection and in all but the mildest cases becoming generalised involving the whole of the somatic muscular system. Most frequently, the disease follows injury, which may even be too trivial to be noticed. Puncture wounds are particularly vulnerable as they favour the growth of the anaerobic bacillus. Rarely, it may follow surgical operations, usually due to lapses in asepsis. Sometimes the disease may be due to local suppuration, such as otitis media (otogenic tetanus). Tetanus is an important complication of septic abortion. It may be caused by dirty practices, such as application of cowdung on the umblical stump or rituals such as earboring or circumcision by unhygienic techniques. Tetanus may also be caused by unsterile injections.

Mere contamination of a wound with tetanus spores does not inevitably lead to tetanus. Unless anaerobic conditions are available, the spores remain dormant. Destruction and necrosis of tissue, lack of drainage in the area, presence of extraneous matter, especially of soil, infection with other bacteria, all favour development of tetanus. Spores may sometimes remain dormant at the site of lodgement for a long time and suddenly erupt into activity either at the original or at another site when conditions become favourable.

The incubation period is variable — from two days to several weeks, but is commonly 6–12 days. This is influenced by several factors, such as the site and nature of the wound, the dose and toxigenicity of the contaminating organism and the immune status of the patient. The incubation period is of prognostic significance — the prognosis being grave when it is short. Of similar significance is the interval between the first symptom of the disease, usually trismus, and the onset of spasms (period of onset).

Tetanus was a serious disease with a high rate of mortality, 80–90 per cent, before specific treatment became available. Even with proper treatment the case fatality rate varies from 15–50 per cent. Tetanus neonatorum and uterine tetanus have very high fatality rates (70–100 per cent), while otogenic tetanus is much less fatal.

Tetanus is more common in the developing countries, where the climate is warm, and in rural areas where the soil is fertile and highly cultı-

vated, where human and animal populations are substantial and live in close association and where unhygienic practices are common and medical facilities poor. In rural India, tetanus is estimated to be the fourth commonest cause of death. In some reports from India, as many as half the tetanus cases are of tetanus neonatorum. The situation calls for mass immunisation, especially of pregnant women.

Laboratory diagnosis: The diagnosis of tetanus should always be made on clinical grounds. Laboratory tests only help in confirmation. Not infrequently, it may not be possible to establish a laboratory diagnosis at all.

Laboratory diagnosis may be made by demonstration of *Cl. tetani* by microscopy, culture or by animal inoculation. Microscopy is unreliable and the demonstration of the typical 'drumstick' bacilli in wounds in itself is not diagnostic of tetanus. The bacilli may be present in some wounds without tetanus developing. It may not also be possible to distinguish by microscopy between *Cl. tetani* and morphologically similar bacilli such as *Cl. tetanomorphum* and *Cl. sphenoides*. Diagnosis by culture is more dependable. Isolation is more likely from excised bits of tissue from the necrotic depths of wounds than from wound swabs. The material is inoculated on one half of a blood agar plate. *Cl. tetani* produces swarming growth which may be detected on the opposite half of the plate after 1–2 days incubation anaerobically. The material is also inoculated into three tubes of cooked meat broth, one of which is heated to 80°C for 15 minutes, the second for five minutes and the third left unheated. The purpose of heating for different periods is to kill vegetative bacteria, while leaving undamaged tetanus spores, which vary widely in heat resistance. The cooked meat tubes are incubated at 37°C and subcultured on one half of blood agar plates daily for upto four days. *Cl. tetani* may be isolated in pure culture by subculturing from the swarming edge of the growth. The incorporation of polymyxin B, to which clostridia are resistant, makes the medium more selective.

For identification and toxigenicity testing, Lowbury and Lilly advocated the use of horse blood agar plates (with 4% agar to inhibit swarming), having tetanus antitoxin (1500 units per ml) spread over one half of the plate. the suspected *Cl. tetani* culture is stab-inoculated on each half of the plate, which is then incubated anaerobically for two days. Toxigenic *Cl. tetani* strains show haemolysis around the colonies only on the half without the antitoxin. Lysis is inhibited by the antitoxin on the other half. This may help in identification of the culture as *Cl. tetani*, but is unreliable as a test of toxigenicity since it indicates the production only of tetanolysin and not necessarily of tetanospasmin, which is the pathogenic toxin.

Toxigenicity is best tested in animals. A two-to-four-day-old cooked meat culture (0.2 ml) is inoculated into the root of the tail of a mouse. A second mouse that has received tetanus antitoxin (1000 units) an hour earlier serves as the control. Symptoms develop in the test animal in 12–24 hours, beginning with stiffness of the tail. Rigidity proceeds to the leg on the inoculated side, the opposite leg, trunk and forelimbs in that order. The animal dies within two days, but may be killed earlier as the appearance of ascending tetanus is diagnostic.

Prophylaxis: Tetanus is a preventible disease. As the spores are ubiquitous, wound contamination is unavoidable. The disease is due to the action of the toxin and, hence, the obvious and most dependable method of prevention is to build up antitoxic immunity by active immunisation.

The nature of prophylaxis depends largely on the type of wound and the immune status of the patient. The available methods of prophylaxis are 1) surgical attention, 2) antibiotics, and 3) immunisation — passive, active or combined.

Surgical prophylaxis aims at removal of foreign bodies, necrotic tissue and blood clots, in order that an anaerobic environment favourable for the tetanus bacillus is not provided. The extent of surgical treatment may vary from simple cleans-

ing to radical excision, depending on the type of wound.

Antibiotic prophylaxis aims at destroying or inhibiting tetanus bacilli and pyogenic bacteria in wounds so that the production of toxin is prevented. In experimentally infected animals, tetanus can be prevented by antibiotics when administered four hours after infection, but not after eight hours. This emphasises the need for prompt administration of antibiotics. Penicillin (given as daily injections or as long-acting preparation) or oral tetracycline may be given till healing is established. Bacitracin or neomycin may be applied locally also. Antibiotics have no action on the toxin. Hence, antibiotic prophylaxis does not replace immunisation, but serves as a useful adjunct.

Passive immunisation with tetanus antitoxin (antitetanus serum, ATS), prepared by immunisation of horses, has proved effective in preventing tetanus. The recommended dose is 1500 International Units administered subcutaneously or intramuscularly as early as possible after wounding. The prophylactic value of ATS was clearly established in wounded soldiers during the 1914–18 war. Not only did ATS bring about a marked reduction in the incidence of tetanus, but when it did not prevent the development of tetanus, it lengthened the incubation period and diminished the fatality.

ATS carries two disadvantages implicit in the use of any heterologous serum — 'immune elimination' and hypersensitivity. The half-life of ATS in man is normally about seven days, but in persons who have had prior injections of horse serum, it is eliminated much quicker by combination with preexisting antibodies. Prior sensitisation also leads to hypersensitivity reactions which may range from mild local reactions to serum sickness, and even fatal anaphylaxis. It is, therefore, obligatory that a test for hypersensitivity should invariably be made before administration of ATS. The intradermal test for hypersensitivity, which is in common use, has been reported to be unreliable. A 'trial' dose given subcutaneously would be a better index of hypersensitivity. A dose of 0.05 ml of ATS is given subcutaneously and the patient observed for at least half an hour for general reactions. As even this dose may precipitate anaphylaxis in some cases, a syringe loaded with adrenaline (1/1000) should invariably be kept ready. In persons with a history of any allergy, the trial dose should be 0.05 ml of a 1/10 dilution of ATS.

Bovine and ovine ATS were introduced to overcome reactions to horse serum, but these in turn can also produce hypersensitivity. Passive immunity without risk of hypersensitivity can be obtained by the use of human antitetanus immunoglobulin (ATG). This is effective in smaller doses (250 units) and has a longer half-life (3–5 weeks). As ATG is prepared by immunisation of human volunteers, its availability will be limited.

It has to be remembered that passive immunisation is an emergency procedure to be used only once. The practice of persons receiving ATS every time they are wounded is not only useless and wasteful, but also positively dangerous. In some countries with a low incidence of tetanus, heterologous ATS is not recommended at all, tetanus being attempted to be controlled by active immunisation, with human ATG being reserved for emergency use in the non-immune.

Active immunisation is not only the most effective method of prophylaxis, but also the only means whereby tetanus following unnoticed injuries can be prevented. This is achieved by spaced injections of formol toxoid, which is available either as 'plain toxoid, or adsorbed on aluminium hydroxide or phosphate. The adsorbed toxoid is a better antigen. Tetanus toxoid is given either alone or along with diphtheria toxoid and pertussis vaccine as 'triple vaccine', in which pertussis vaccine acts as an adjuvant. A course of immunisation consists of three doses of tetanus toxoid given intramuscularly, with an interval of 4–6 weeks between the first two injections and 6–12 months

between the second and third injections. A full course of immunisation confers immunity for a period of at least ten years. A 'booster dose' of toxoid is recommended after ten years. ATS should not be given to an immunised individual. Instead, a booster dose of toxoid is given if wounding occurs three years or more after the full course of immunisation. Too frequent injection of toxoid should be avoided lest hypersensitivity reactions develop.

In World War II, only 12 cases of tetanus occurred in 2,734,819 hospital admissions for wounds or injuries in the American soldiers who had been previously immunised.

Combined immunisation consists of administering to a nonimmune person ATS in one site, along with the first dose of toxoid at another site, followed by the second and third doses of toxoid at appropriate intervals. It is important to use adsorbed toxoid as the immune response to plain toxoid may be interfered with by ATS. Ideally, combined immunisation should be routinely performed instead of passive immunisation alone. This obviates the need for further ATS if the person gets wounded again.

Table 28.3 shows the recommended integrated prophylaxis of tetanus following injury.

Treatment: Tetanus patients should be treated in hospitals, preferably in special units. The reason for isolating them is to protect them from noise and light which may provoke convulsions. But, because they are isolated, there is a common impression that they are highly infectious. This is not true. Tetanus patients are hardly ever infectious, and person to person transmission does not occur at all.

Treatment consists of ensuring quiet, controlling spasms, maintaining airway by tracheostomy with intermittent positive pressure respiration and attention to feeding. ATS used to be given intravenously in massive doses as part of the treatment. Doubts have been expressed recently on the therapeutic value of antitoxin, as once tetanus is established the antitoxin is unable to neutralise the toxin already fixed to the nervous tissue. On the other hand, the antitoxin is able to neutralise unbound toxin and any further toxin that may be produced. Several controlled trials have been undertaken to assess the value of antitoxin and the optimum dose. The results indicate that ATS is of value in treatment, but that 10,000 I.U. intravenously gives as good results as much higher doses.

Patients recovering from tetanus should receive a full course of active immunisation, as an attack of the disease does not confer immunity. Second attacks of tetanus have been recognised and when this occurs, therapeutic ATS may not be applicable due to prior sensitisation.

CLOSTRIDIUM BOTULINUM

Cl. botulinum causes botulism, a severe form of food poisoning. The name botulism is derived from sausage (*botulus*, Latin for sausage), an article of food that used to be associated with this type of food poisoning. *Cl. botulinum* was first isolated by van Ermengem (1896) from a piece of ham that caused an outbreak of botulism. The bacillus is a widely distributed saprophyte, occurring in virgin soil, vegetables, hay, silage, animal manure and sea mud.

Morphology: It is a Gram positive bacillus about $5\mu \times 1\mu$, noncapsulated, motile by peritrichate flagella, producing subterminal, oval, bulging spores.

Cultural characteristics: It is a strict anaerobe. Optimum temperature is 35°C, but some strains may grow even at 1°-5°C. Good growth occurs on ordinary media. Surface colonies are large, irregular, semitransparent, with fimbriate border. Biochemical reactions vary in different types. Spores are produced consistently when grown in alkaline glucose gelatin media at 20°C to 25°C. They are not usually produced at higher temperatures.

Resistance: Spores are highly resistant, surviving

TABLE 28.3

Tetanus prophylaxis in the wounded

Nature of wound	*Immune status of the person*		
	Immune	*Partially immune*	*Non-immune*
Clean (wound toilet performed within 6 hours)	Toxoid x 1	Toxoid x 1	Toxoid x 3
Contaminated (soil or other foreign or necrotic material present)	Toxoid x 1	Toxoid x 1 ATS Antibiotics	Toxoid x 3 ATS Antibiotics
Infected	Toxoid x 1 Antibiotics	Toxoid x 1 ATS Antibiotics	Toxoid x 3 ATS Antibiotics

Note: Immune—Patient has had a full course of three injections of toxoid.
Partially immune—Patient has had two injections of toxoid.
Nonimmune—Patient has had one or no injection of toxoid, or immunisation status is not known.

several hours at 100°C and for upto 20 minutes at 120°C. Spores of nonproteolytic types of B, E and F are much less resistant to heat.

Classification: Eight types of *Cl. botulinum* have been identified (Types A to H), based on the immunological difference in the toxins produced by them. The toxins produced by the different types are identical in their pharmacological activity, but are neutralised only by the homologous antiserum. Until recently, it was believed that any individual strain produced only one type of toxin but a strain has been isolated which produces both A and F toxins.

Toxin: *Cl. botulinum* produces a powerful exotoxin that is responsible for its pathogenicity. The toxin differs from other exotoxins in that it is not released during the life of the organism. It is produced intracellularly and appears in the medium only on the death and autolysis of the cell. It is believed to be synthesised initially as a nontoxic protoxin or progenitor toxin. Trypsin and other proteolytic enzymes activate progenitor toxin to active toxin.

The toxin has been isolated as a pure crystalline protein, which is probably the most toxic substance known. It has a M.W. 70,000 and a lethal dose for mice of 0.000,000,033 mg. The lethal dose for humans is probably 1–2 μg. It is a neurotoxin and acts slowly, taking several hours to kill.

The toxin is relatively stable, being inactivated only after 30–40 minutes at 80°C and 10 minutes at 100°C. Food suspected to be contaminated with botulinum toxin can be rendered completely safe by pressure cooking or boiling for 20 minutes. It resists digestion and is absorbed through the small intestines in an active form. It acts by blocking the production or release of acetylcholine at synapses and neuromuscular junctions. This leads to diplopia, dysphagia, respiratory paralysis and other motor paralysis. Death occurs due to respiratory failure.

Botulinum toxin can be toxoided. It is specifically neutralised by its antitoxin and is a good antigen. The toxins produced by the different types of *Cl. botulinum* appear to be identical, except for immunological differences. Toxin production appears to be determined by the presence of bacteriophages, at least in types C and D.

Pathogenicity: Cl. botulinum is noninvasive and virtually noninfectious. Its pathogenicity is due to the action of its toxin, the manifestations of which are collectively called botulism. Botulism is of three types — foodborne, wound, and infant.

Foodborne botulism is due to the ingestion of preformed toxin. The types of the bacillus and the nature of the food responsible vary in different regions. Human disease is usually caused by types A, B, E and very rarely F. Types C and D are usually associated with outbreaks in cattle and wild fowl. Type G, isolated from soil in Argentina, has not yet been associated with disease. The source of botulism is usually preserved food — meat and meat products in Europe, canned vegetables in America and fish in Japan. Type E is associated with fish and other seafoods. The food responsible for botulism usually exhibits signs of spoilage, and cans are often inflated and show bubbles on opening, though at times the food may look normal.

Symptoms begin usually 12–36 hours after ingestion of the food. Vomiting, thirst, constipation, ocular pareses, difficulty in swallowing, speaking and breathing constitute the common features. Coma or delirium may supervene. Death is due to respiratory failure and occurs 1–7 days after onset. Case fatality varies from 25–70 per cent.

Wound botulism is a very rare condition resulting from wound infection with *Cl. botulinum*. Between 1943 and 1974, fifteen cases were reported in the United States, four of them fatal. Toxin is produced at the site of infection and is absorbed. The symptoms are those of foodborne botulism except for the gastrointestinal components which are absent. Type A has been responsible for most of the cases studied.

Infant botulism was recognised as a clinical entity in 1976. This is a toxico-infection. *Cl. botulinum* spores are ingested in food, get established in the gut and there produce the toxin. Cases occur in infants below six months. Older children and adults are not susceptible. The manifestations are constipation after a period of normal development, poor feeding, lethargy, weakness, pooled oral secretions, weak or altered cry, floppiness and loss of head control. Patients excrete toxin and spores in their faeces. Toxin is generally not demonstrable in blood. Management consists of supportive care and assisted feeding. Antitoxins and antibiotics are not indicated. Degrees of severity vary from very mild illness to fatal disease. Some cases of sudden infant death syndrome have been found to be due to infant botulism. Honey has been incriminated as the likely food item through which the bacillus enters the gut.

Laboratory diagnosis: Diagnosis may be confirmed by demonstration of the bacillus or the toxin in food or faeces. Gram positive sporing bacilli may be demonstrable in smears made from food. *Cl. botulinum* may be isolated from the food or the patient's faeces. The food is macerated in sterile saline, and the filtrate inoculated into mice or guinea pigs intraperitoneally. Control animals protected by polyvalent antitoxin remain healthy. Typing is done by passive protection with type specific antitoxin. The toxin may occasionally be demonstrable in the patient's blood, or in the liver, post mortem.

A retrospective diagnosis may be made by detection of antitoxin in the patient's serum, but it may not be detectable in all cases.

Control: As most cases of botulism follow consumption of inadequately canned or preserved food, control can be achieved by proper canning and preservation. When an outbreak occurs, a prophylactic dose of antitoxin should be given

intramuscularly to all who consumed the article of food.

Active immunisation has been shown to be effective. If immunisation is needed, as in laboratory workers exposed to the risk, two injections of aluminium sulphate adsorbed toxoid may be given at an interval of ten weeks, followed by a booster a year later. Antitoxin may be tried for treatment. Polyvalent antiserum to types A, B and E may be administered as soon as a clinical diagnosis is made. Supportive therapy with maintenance of respiration is of equal or greater importance. In addition, a combination of guanidine hydrochloride and germine monoacetate may be administered bearing in mind their potential toxicity and side effects

CLOSTRIDIUM DIFFICILE AND ANTIBIOTIC-ASSOCIATED COLITIS

Cl difficile was first isolated in 1935 from the faeces of newborn infants. It was so named because of the unusual difficulty in isolating it. It is a long, slender, Gram positive bacillus with a pronounced tendency to lose its Gram reaction. Spores are large, oval and subterminal. It is non-haemolytic, nonproteolytic and weakly saccharolytic. It was not considered pathogenic till 1977, when it was found to be responsible for antibiotic associated colitis.

Acute colitis, with or without membrane formation, is an important complication of oral antibiotic therapy. Many antibiotics have been incriminated including ampicillin, tetracycline and chloramphenicol, but lincomycin and clindamycin are particularly prone to cause pseudomembranous colitis.

It has now been shown that antibiotic-associated colitis is due to the active multiplication of *Cl. difficile* and the production by it of an enterotoxin. Diagnosis can be made by demonstrating the toxin in the faeces of patients by its characteristic effect of Hep-2 and human diploid cell cultures. The toxin is specifically neutralised by *Cl. sordelli* antitoxin. *Cl. difficile* can also be grown from the faeces of patients.

Cl difficile strains are generally resistant to most antibiotics, but are susceptible to Vancomycin, which has been very successful in the treatment of antibiotic-associated colitis. Metronidazole has also been found useful.

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29 Nonsporing anaerobes

Anaerobic bacteria have been known since the original observation of Pasteur that bacteria producing butyric acid, his *Vibrion butyrique*, were rendered nonmotile on exposure to air. Though many anaerobic bacteria may be pathogenic for man, they are generally neglected in diagnostic laboratories. This neglect is not because they are uncommon. Indeed, they outnumber aerobic bacteria in many habitats, including most sites of the human or animal body. Even in such seemingly aerobic situations as the mouth and the skin, anaerobic bacteria are ten to thirty times more frequent than aerobes. In the human intestines, they outnumber aerobic bacteria a thousandfold. The numbers of anaerobes present have been estimated to be 10^4 to 10^5/ml in the small intestine, 10^9/ml in saliva and 10^{11}/g in colon.

Anaerobic bacteria differ widely in the degree of anaerobiosis required for their growth. Some species fail to grow if the atmosphere contains as little as 0.03 per cent oxygen, while at the other extreme, some are aerotolerant and may grow sparsely on the surface of aerobic plates. Consequently the techniques employed for the propagation and study of anaerobes would vary in complexity. The classification of anaerobes is based on morphology and staining characters, biochemical reactions, antibiotic susceptibility patterns and metabolic products. Anaerobic bacteria form certain volatile fatty acids as metabolic products. These are characteristic for different species and can be detected by gas liquid chromatography. Examination of pus by gas liquid chromatography gives a presumptive indication of the types of anaerobes present.

The classification of anaerobic bacteria is unsettled, different systems and nomenclature being employed by different authorities. A broad and simple classification based on their morphology and staining properties is as follows:

I *Cocci*
- A. Gram positive
  1 *Peptococcus*
  2 *Peptostreptococcus*
  3 *Sarcina*
- B. Gram negative
  1 *Veillonella*

II *Bacilli*–Endospore-forming–*Clostridium*
Nonsporing
- A. Gram positive
  1 *Lactobacillus*
  2 *Bifidobacterium*
  3 *Propionibacterium*
  4 *Actinomyces*
- B. Gram negative
  1 *Bacteroides*
  2 *Fusobacterium*
  3 *Dialister*
  4 *Sphaerophorus*

III *Spirochaetes*
  1 *Treponema*
  2 *Borrelia*

Besides the medically important species listed above, there are several anaerobes that occur in soil and water and which may be of industrial and agricultural importance (e.g. Methanobacteria, Butyrivibrios).

Anaerobic cocci

Veillon (1983) isolated a strictly anaerobic coccus

from cases of bartholinitis and perinephric abscess. As the cultures produced a foul odour, the isolate was named *Micrococcus foetidus*. Anaerobic cocci occurring mainly in chains were isolated soon afterwards by other workers from the normal vagina and from cases of puerperal sepsis. The importance of anaerobic streptococci in puerperal sepsis and septicaemia was recognised by Schottmueller who named the causative organism *Str. putridus*.

Anaerobic cocci represent a heterogeneous collection of bacteria for which a satisfactory system of classification and nomenclature has not been devised. Prevot, who presented the first systematic classification, proposed two families, *Neisseriaceae* and *Micrococcaceae* to include the Gram negative and Gram positive cocci, respectively, which were further divided into three tribes, 10 genera and 41 species. Hare and colleagues classified them into nine groups, Group I representing *Str. putridus* and Group V *Veillonella*. In Bergey's Manual (8th Edition), the classification is based on Gram reaction and arrangement of the cocci. They are classified into *Peptococcus*, *Peptostreptococcus*, *Sarcina* and *Veillonella*.

Peptococci are Gram positive anaerobic cocci that do not form chains. They are small spherical cells, 0.5–1 μ in size, arranged singly, in pairs or irregular masses, resembling staphylococci. They are coagulase negative. They utilise protein decomposition products like peptone and aminoacids. Carbohydrates are not required for growth.

Peptostreptococci are small Gram positive cells, 0.3–1 μ in size, arranged in chains. Carbohydrates are fermented with the production of acid, gas or both. The commonest anaerobic coccus isolated from clinical materials is *Peptostreptococcus putridus (P. anaerobius)*.

Sarcina are large, spherical, Gram positive cells, about 2–3 μ in size, occurring in packets of eight or more. Though the original species *S. ventriculi* was isolated from the stomach of a patient with gastric disease, sarcinae are nonpathogenic and are normally found in soil.

Veillonellae are Gram negative, small cells, 0.3–0.5 μ in size, occurring as diplococci, masses or short chains. Their pathogenic status is doubtful.

Anaerobic cocci are normal inhabitants of the vagina, intestines and mouth. Peptococci and peptostreptococci are potential pathogens, causing pyogenic infections in different sites in the body. They have been isolated from puerperal sepsis, wound infections, gangrenous appendicitis, urinary tract infections, osteomyelitis and abscesses in the brain, lungs and other internal organs.

P. putridus is the commonest agent of postpartum and postabortal infections today. Such infections are endogenous and are not transmitted from case to case as were the epidemics of puerperal sepsis caused by *Str. pyogenes* in former days.

Anaerobic cocci are frequently associated with clostridia in wound infections and anaerobic cellulitis. Anaerobic streptococci are sometimes isolated in pure culture from sexually acquired suppurative balanitis. In suppurative lesions, such as brain abscess, very large numbers of the cocci are present in the pus, so that examination of the Gram stained films of the pus may indicate the aetiology. Anaerobic cocci are sensitive to penicillin, tetracyclines and chloramphenicol and resistant to streptomycin and polymyxin.

Anaerobic Gram negative bacilli

A simple classification of this group was adopted in the 7th edition of Bergey's Manual. They were grouped in the family *Bacteroidaceae* and classified into four genera: 1) *Bacteroides*, bacilli with rounded ends, 2) *Fusobacterium*, bacilli with pointed ends, 3) *Dialister*, minute bacilli, and 4) *Sphaerophorus*, markedly pleomorphic cells.

Bacteroides are normal inhabitants of the upper respiratory, intestinal and female genital tracts. Normal stools contain 10^{11} organisms per gram. The most common are *B. fragilis* group (five species) isolated particularly from the large intestine, *B. melaninogenicus* (seven species) isolated particularly from the oropharynx, gut and

vagina, *B. bivius* and *B. disiens* occurring in the female genital tract.

These are nonsporing, nonmotile, strict anaerobes, usually Gram negative and very pleomorphic, appearing as slender rods, branching forms or round bodies. They grow very readily in media such as brain-heart infusion agar in an atmosphere containing 10% carbon dioxide. They possess capsular polysaccharides which are their virulence factors, and antibodies to them can be detected in patients.

Bacteroides are found in abdominal and brain abscesses and in empyema. They cause suppuration in surgical infections such as peritonitis following bowel injury and pelvic inflammatory disease (PID). Pus is often foul smelling. Bacteraemia is common and endocarditis may develop. In these infections other anaerobic organisms such as *Peptostreptococcus, Peptococcus, Clostridia, Fusiformis* as well as Gram positive and Gram negative facultative anaerobes that are often part of the normal flora are often associated.

The genus *Bacteroides* contains several well defined species *B. fragilis* is probably the most frequent among nonsporing anaerobes isolated from clinical specimens. It is often isolated from blood, pleural and peritoneal fluid, CSF, brain abscess, wounds and urogenital infections. *B. melaninogenicus* is easy to recognise because of the black or brown colour of the colonies. The colour is not caused by a melanin pigment, as was once thought, but is due to a haemin derivative. It has been isolated from various mixed infections, including lung or liver abscess, mastoiditis, intestinal lesions of various types and lesions of the mouth and gums. Cultures of *B. melaninogenicus* and even dressings from wounds infected with the bacillus give a characteristic red fluorescence when exposed to ultraviolet light.

F. fusiformis has been frequently isolated from blood, CSF, bone marrow and abscesses in internal organs. *F. gyrans* is characterised by a gyratory or spinning type of motion. It has been isolated from wound infections and peritoneal abscess. *Sphaerophorus necorphorus* is very pleomorphic bacillus. It has been divided into two types based on colony and cell morphology, biochemical reactions and toxin production. Type A strains are more pathogenic and have been isolated from man, rabbits and cattle.

Differentiation between fusobacteria and sphaerophora is often difficult and it has been suggested that they be considered as a single genus *Fusobacterium*.

Dialister pneumosintes is a minute, Gram negative rod, first isolated from the nasal washings of influenza patients. It is a normal inhabitant of the nasopharynx. It is so small (0.1-0.3μ) that it can pass through Berkfeld V and N filters. It has been isolated from brain abscess and meningitis.

Anaerobic Gram negative bacilli are usually sensitive to pencillin, tetracycline and chloramphenicol and resistant to streptomycin, kanamycin and oleandomycin. *B. fragilis* is more resistant than other species.

Anaerobic Gram positive bacilli

Lactobacilli are Gram positive rods that frequently show bipolar and barred staining resembling corynebacteria. Most strains are nonmotile. They form considerable amounts of lactic acid from carbohydrates, are aciduric and grow best at a pH of 5 or less. Some species have strict growth requirements and are used for the microbiological assay of the growth factors (e.g., *L. leichmannii* used for the assay of vitamin B_{12}).

Lactobacilli are normally present in the mouth They have been incriminated in the pathogenesis of dental caries. The mineral components of enamel and dentine are believed to be dissolved by the acid formed by the fermentation of sucrose and other dietary carbohydrates.

Several species of lactobacilli are present in the intestine, the commonest being *L. acidophilus*. Intestinal lactobacilli are beneficial in synthesising vitamins such as biotin, vitamin B_{12} and vitamin K, which may be absorbed by the host.

Lactobacilli constitute the predominant bacterial flora of the adult vagina. They ferment the

glycogen deposited in the vaginal epithelial cells, forming lactic acid, which accounts for the highly acid pH of the vagina. Lactobacilli of several species occur in the vagina and are collectively known as the 'Doderlein's bacillus'. In prepubertal and postmenopausal vagina, lactobacilli are scanty.

Bifidobacteria are related to lactobacilli and corynebacteria. The name is derived from the frequent bifid Y-shaped cells. They are normal intestinal commensals and are occasionally pathogenic.

Propionibacteria are related to corynebacteria and consist of the organisms usually labelled *'anaerobic diptheroids'*. The commonest species met with in the clinical laboratory is *P. acnes (Corynebacterium acnes)*. This is n ubiquitous commensal found abundantly on the normal skin. Its pathogenic role is not definite. It is regularly found in acne and was thought to be its causative agent and hence the name. It has also been isolated from abscesses, blood and bone marrow. It is a common contaminant in bank blood. The antibody to this bacillus is frequently found in normal human sera.

Actinomycetes are dealt with in Chapter 43.

Anaerobic Infections

There has been a reawakening of interest in anaerobic infections during recent years. This is due to the availability of improved and simplified techniques for the isolation and identification of anaerobes.

Anaerobic infections are usually endogenous and are caused by tissue invasion by bacteria normally resident on the respective body surfaces. It is, therefore, necessary to know the distribution of anaerobes on the body surfaces. Anaerobic bacteria are normally present on the skin, mouth, nasopharynx and upper respiratory tract, intestines and vagina (Table 29.1). Anaerobic infections generally follow some precipitating factor such as trauma, tissue necrosis, impaired circulation, haematoma formation or presence of foreign body. Diabetes, malnutrition, malignancy or prolonged treatment with aminoglycoside antibiotics may act as predisposing factors. Anaerobic infections are typically polymicrobial, more than one anaerobe being responsible, besides aerobic bacteria. While the infection is usually localised, general dissemination may occur by bacteraemia. Table 29.2 lists the common sites and type of anaerobic infections and the bacteria responsible.

There are some clinical features which suggest the presence of anaerobic infection. Pus produced by anaerobes is characteristically putrid, with a pervasive, nauseating odour. However, there may be exceptions; infections due solely to *B. fragilis* have been reported to be free of this

TABLE 29.1
Normal anaerobic flora of the human body

Anaerobe	Skin	Mouth, Nasopharynx	Intestine	Vagina
Clostridium			++	
Actinomyces		+		
Bifidobacterium		+	++	+
Propinibacterium	++			
Bacteroides fragilis			++	
B. melaninogenicus		++	+	++
Fusobacteria		++	+	
Gram positive cocci		++	++	++
Gram negative cocci		++	+	++
Spirochaetes		+		

smell. Pronounced cellulitis is a common feature of anaerobic wound infections. Toxaemia and fever are not marked.

Laboratory diagnosis

As anaerobes form part of the normal flora of the skin and mucous surfaces, their isolation from specimens has to be interpreted cautiously. The mere presence of an anaerobe does not prove its causal role. Specimens should be collected in such a manner as to avoid resident flora. For example, the sputum is unsatisfactory for culture from a suspected case of lung abscess; only material collected by aspiration would be acceptable.

As some anaerobes die on exposure to oxygen, care should be exercised to minimise contact with air during collection, transport and handling of specimens. A satisfactory method of collection is to aspirate the specimen into an airtight syringe, plunge the needle into a sterile rubber cork to seal it and send it as such immediately to the

TABLE 29 2

Common anaerobic infections and the bacteria responsible

Site and type of infection	Bacteria commonly responsible
Central Nervous System:	
Brain abscess	*B fragilis, Peptostreptococcus*
Ear, Nose, Throat.	
Chronic sinusitis, otitis media, mastoiditis, orbital cellulitis	Fusobacteria (aerobes frequently responsible)
Mouth and Jaw:	
Ulcerative gingivitis (Vincent's)	Fusobacteria, spirochaetes
Dental abscess, cellulitis–	Mouth anaerobes.
Abscess and sinus of jaw	Actinomyces, other mouth anaerobes.
Respiratory.	
Aspiration pneumonia, lung abscess, bronchiectasis, empyema.	Fusobacteria, *B. melaninogenicus*, anaerobic cocci; *B. fragilis* rarely
Abdominal.	
Subphrenic, hepatic abscess; appendicitis, peritonitis; ischiorectal abscess; wound infection after colo-rectal surgery.	*B. fragilis*
Female genitalia:	
Wound infection following genital surgery; Puerperal sepsis; tubo-ovarian abscess, Bartholin's abscess	*B. melaninogenicus*, anaerobic cocci; *B fragilis*
Septic abortion	Genital anaerobes and *Cl. welchii.*
Skin and soft tissue:	
Infected sebacious cyst	Anaerobic cocci.
Breast abscess, axillary abscess	Anaerobic cocci, *B. melaninogenicus* (*Staph aureus* commonest cause)
Cellulitis, diabetic ulcer, gangrene.	*B. fragilis* and others.

laboratory. Pus and other fluids may be collected in small bottles with airtight caps and transported quickly, ensuring that the specimens fill the bottles completely. Swabs are generally unsatisfactory, but where they are to be used, they should be sent in Stuart's transport medium.

In the laboratory, exposure should be limited to the minimum. Examination of a Gram stained smear is useful. Pus in anaerobic infection usually shows a large variety of different organisms and numerous pus cells. Rarely, as in brain abscess, a single type of organism alone may be seen. Examination of the specimen under ultraviolet light may show the bright red fluorescene of *B. melaninogenicus*. Gas liquid chromatography of the specimen may yield presumptive information on the types of anaerobes present.

Several special media have been described for anaerobes, but for routine diagnostic work, freshly prepared blood agar with neomycin, yeast extract, haemin and vitamin K is adequate. Plates are incubated at 37°C in an anaerobic jar, with 10 per cent CO_2. The Gas-Pak system provides a convenient method of routine anaerobic cultures. Plates are examined after 24 or 48 hours. Some anaerobes, such as fusobacteria, require longer periods of incubation. Parallel aerobic cultures should always be set up. This is necessary as a control for the growth on anaerobic plates and also because in most anaerobic infections aerobic bacteria are also involved.

Sophisticated methods, such as the use of 'gassed' containers, gloved anaerobic chambers with continuous gas flow and prereduced, anaerobically sterilised media, are employed for quantitative studies, but are not essential for routine diagnostic practice.

Definitive identification of the anaerobes isolated may take time and prove difficult, but it should be possible within two or three days to report on the following: 1) whether the infection is solely aerobic, anaerobic or mixed, 2) the identity of the commoner anaerobes, particularly of *B. fragilis*, which is the commonest anaerobe isolated from clinical materials and the most resistant to antibiotics, and 3) an indication of the antimicrobial agent likely to be useful. Sensitivity tests can be done, if desired, by the disc or dilution method.

Treatment

Some anaerobic infections such as Vincent's angina can be managed entirely by antimicrobial therapy, but most infections require surgical intervention also. Penicillin is effective against most anaerobes, with the notable exception of *B. fragilis*. Tetracycline and chloramphenicol have been used with good results, but strains resistant to chloramphenicol are now common. Aminoglycoside antibiotics, and gentamycin in particular, are ineffective against anaerobes. Clindamycin is effective, but is not commonly used at present because it is prone to cause pseudomembranous colitis. The drug of choice now is metronidazole. It has also been found useful as a prophylactic in large bowel surgery.

Further Reading

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30 Enterobacteriaceae—I Coliforms: Proteus

The predominant aerobic bacterial flora of the large intestines of man and animals is composed of nonsporing, nonacid fast, Gram negative bacilli. They exhibit general morphological and biochemical similarities and are grouped together in the large and complex family *Enterobacteriaceae*. Members of this family may or may not be capsulated and are motile by peritrichate flagella, or nonmotile. They are aerobic and facultatively anaerobic, grow readily in ordinary media, ferment glucose producing acid and gas or acid only, reduce nitrates to nitrites and form catalase, but not oxidase. Within the family, they exhibit very wide biochemical and antigenic heterogeneity. Though the family is subdivided into groups or tribes, genera, subgenera, species and types, many strains are met with that possess every conceivable combination of characters and do not fall into any such arbitrary taxonomic category. The frequency of genetic mechanisms such as conjugation and transduction in these bacteria contributes to their infinite variety. Classification of these bacteria into well demarcated compartments, though necessary for their systematic study, would therefore be artificial. The classification of *Enterobacteriaceae* has been controversial, and there have been successive changes in their grouping and nomenclature.

The oldest method was to classify these bacteria into three groups based on their action on lactose:

I. Lactose fermenters – e.g., *Escherichia*, *Klebsiella*

II. Late lactose fermenters – e.g., *Shigella sonnei*, *'Paracolons'*

III. Nonlactose fermenters – e.g., *Salmonella*, *Shigella*

This method of classification was derived from the use of lactose in the MacConkey's medium, the most popular medium for the isolation of faecal bacilli. Though taxonomically unacceptable, this scheme had practical value in diagnostic bacteriology. The majority of the commensal intestinal bacilli are lactose fermenting. As the most common member of this group is the 'colon bacillus' or *Escherichia coli*, all lactose fermenting enteric bacilli were called *coliform bacilli*. The major intestinal pathogens, *Salmonella* and *Shigella* are nonlactose fermenters, and hence readily detectable by the colourless colonies they form on MacConkey's medium. There remained a small group which showed delayed fermentation of lactose and with the exception of *Shigella sonnei*, they were all commensals. This heterogenous group of late lactose fermenters was called *paracolon bacilli*.

Classification based on a single property, such as lactose fermentation, is contrary to modern taxonomical concepts. The current practice is to group together bacteria that possess a number of common morphological and biochemical properties. While the three widely used systems for the classification of *Enterobacteriacea* (Bergey's manual; Kauffmann; Edwards-Ewing) feature certain differences, the general approach is the same. The family is first classified into its major subdivision—group or tribe. Each tribe consists of one or more genera and each genus, one or more subgenera and species. The species are clas-

sified into types — biotypes, serotypes, bacteriophage types, colicin types

Enterobacteriaceae

Tribe I – *Eschericheae*
Genus 1. *Escherichia*
2. *Edwardsiella*
3. *Citrobacter*
4. *Salmonella*
5. *Shigella*
Tribe II – *Klebsielleae*
Genus 1. *Klebsiella*
2. *Enterobacter*
3. *Hafnia*
4. *Serratia*
Tribe III – *Proteeae*
Genus 1. *Proteus*
Tribe IV – *Erwinieae*
Genus 1. *Erwinia*

The genus *Yersinia*, including the plague bacillus, has been recently placed in the family *Enterobacteriaceae*, but because of the special importance of plague and its lack of similarity to 'enteric' disease, it is dealt with separately. The genus *Erwinia* is not considered as it consists of plant pathogens and commensals of no medical importance.

Table 30.1 lists the important features of the different genera

ESCHERICHIA COLI

This genus is named after Escherich who was the first to describe the colon bacillus under the name *Bacterium coli commune* (1885). Based on minor differences in biochemical characteristics, colon bacilli were described under various names, but in view of the mutability of the biochemical properties in this group, only one species is recognised in this genus — *Escherichia coli*, which is further subdivided into biotypes and serotypes.

Unlike other coliforms, *E. coli* is a parasite living only in the human or animal intestine. Voided in faeces, it remains viable in the environment only for some days. Detection of *E. coli* in drinking water, therefore, is taken as evidence of recent pollution with human or animal faeces.

Morphology

E. coli is a Gram negative straight rod, measuring 1–3μ x 0.4–0.7μ arranged singly or in pairs. It is motile by peritrichate flagella, though some strains may be nonmotile. Capsules and fimbriae are found in some strains. Spores are not formed.

Cultural characteristics

It is an aerobe and a facultative anaerobe. The temperature range is 10–46°C (optimum 37°C). Good growth occurs on ordinary media. Colonies are large, thick, greyish white, moist, smooth, opaque or partially translucent discs. This description applies to the 'smooth' (S) form seen on fresh isolation, which is easily emulsifiable in saline. The 'rough (R) forms give rise to colonies with an irregular dull surface and are often autoagglutinable in saline. The S→R variation occurs as a result of repeated subcultures and is associated with the loss of surface antigens and, usually, of virulence. Some strains may occur in the 'mucoid' form.

Many strains, especially those isolated from pathological conditions, are haemolytic on blood agar. On MacConkey's medium, colonies are bright pink due to lactose fermentation. Growth is largely inhibited on selective media such as DCA or SS agar used for the isolation of salmonellae and shigellae. In broth, growth occurs as general turbidity and a heavy deposit, which disperses completely on shaking.

Biochemical reactions

Glucose, lactose, mannitol, maltose and many other sugars are fermented with the production of acid and gas. Typical strains do not ferment sucrose. The four biochemical tests widely employed in the classification of enterobacteria are the indole, methyl red (MR), Voges-Proskauer (VP) and citrate utilisation tests, generally

referred to by the mnemonic 'IMViC'. *E. coli* is indole and MR positive and VP and citrate negative (IMViC++−−). Gelatin is not liquefied, H_2S is not formed, urea is not split and growth does not occur in KCN medium.

Antigenic structure

Serotyping of *E. coli* is based on three antigens—the somatic antigen O, the capsular antigen K and the flagellar antigen H. So far 164 types of O antigens, 103 K antigens and 75 H antigens have been recognised. The antigenic pattern of a strain is recorded as the number of the particular antigen it carries, as for example 0111: K58: H2.

The K antigen is the 'envelope' antigen located in the capsule or microcapsule. It encloses the O antigen and renders the strain inagglutinable by the O antiserum. It may also contribute to virulence by inhibiting phagocytosis. Three kinds of K antigens, L, A and B, have been described, only one of them being present on any one strain. The L antigen is thermolabile and boiling the bacterial suspension renders it agglutinable with the O antiserum. The A antigen is thermostable. It is usually associated with a well marked capsule, and Quellung reaction may be demonstrated by mixing with the appropriate serum. The B antigen is heat labile. It is medically important as the type of antigen found on enteropathogenic strains of *E. coli*.

Several different serotypes of *E. coli* are found in the normal intestine. Most of them do not have K antigens. Of the strains that possess K antigens, most are of the L type. The normal colon strains belong to the 'early' O groups (1, 2, 3, 4, etc.).

TABLE 30.1

Enterobacteriaceae: Important distinguishing features of the different genera

	Escherichia	Edwardsiella	Citrobacter	Salmonella[1]	Shigella[2]	Klebsiella	Enterobacter	Hafnia	Serratia	Proteus[3]
Motility	+	+	+	+	−	−	+	+	+	+
Gas from glucose	+	+	+	+	−	+	+	+	d	d
Acid from lactose	+	−	+	−	−	+	+	−	−	−
Acid from sucrose	d	−	d	−	−	+	+	−	+	d
Growth in KCN	−	−	+	d	−	+	+	+	+	+
Indole	+	+	d	−	d	−	−	−	−	d
MR	+	+	+	+	+	−	−	−	−	d
VP	−	−	−	−	−	+	+	+	+	d
Citrate	−	−	+	+	−	+	+	+	+	d
H_2S	−	+	+	+	−	−	−	−	−	d
Urease	−	−	−	−	−	+	d	−	−	+
Phenylalanine deaminase (PPA)	−	−	−	−	−	−	−	−	−	+
Arginine dihydrolase	d	−	d	+	−	−	d	−	−	−
Lysine decarboxylase	+	+	−	+	−	d	d	+	+	d
Ornithine decarboxylase	d	+	d	+	d	−	+	+	+	d

(d—results different in different species or strains)

Important exceptions:

1. *S. typhi* does not produce gas from sugars.
2. *Sh. sonnei* ferments lactose and sucrose late.
3. *Pr. inconstans* is urease negative.

while the enteropathogenic strains belong to the 'later' O groups (26,55,86,111,etc).

Toxin production

As in the case of other Gram negative bacteria, *E. coli* also possesses endotoxic activity associated with the O antigen. Besides the endotoxin, some strains of *E. coli* have been found to produce two types of exotoxins — enterotoxins and haemolysins.

Enterotoxins: Some strains of *E. coli* which produce diarrhoea form enterotoxins. Two types of *E. coli* enterotoxins have been identified — heat labile and heat stable. A strain of *E. coli* may produce either one or both types of enterotoxins. Enterotoxin production is under the genetic control of transmissible plasmids (*ent* plasmids).

The heat labile toxin (LT) is a large molecular weight (5×10^6) protein which is inactivated by heating at 60°C in 10 minutes. It resembles the cholera enterotoxin antigenically as well as in its mechanism of action in producing fluid accumulation in the intestinal lumen by stimulating the adenyl cyclase — cyclic adenosine monophosphate (cAMP) system. The heat stable toxin (ST) is a low molecular weight, nonantigenic toxin which appears to stimulate fluid secretion in the gut through the mediation of cyclic guanosine monophosphate (cGMP). In the ligated ileal loop ST induces fluid accumulation more rapidly than LT.

Haemolysins: Three types of haemolysins are found to be produced by *E. coli*. They do not appear to be relevant in pathogenesis.

Pathogenicity

Four main types of clinical syndromes are caused by *E. coli:* 1) urinary tract infection, 2) diarrhoea or gastroenteritis, 3) pyogenic infections, and 4) septicaemia.

1. Urinary tract infection: E. coli and other coliforms account for the large majority of naturally acquired urinary tract infections. Those acquired in the hospital, following instrumentation, are usually caused by other bacteria such as *Pseudomonas* and *Proteus*.

The *E. coli* serotypes commonly responsible for urinary tract infections are those normally found in the faeces, O groups 1, 2, 4, 6, 7 etc. Only one serotype is generally isolated from infected urine at a time, though recurrences may be due to different serotypes.

Infection may be precipitated by urinary obstruction due to prostatic enlargement, calculi or pregnancy. About 5–7 per cent of pregnant women have been reported to have urinary infection without any symptoms. Such *asymptomatic bacteriuria*, undetected and untreated, may lead to symptomatic infection later in pregnancy, pyelonephritis and hypertension in the woman, as well as to prematurity and perinatal death of the fetus.

While infections of the lower urinary tract seem to be 'ascending infection' caused by faecal coliforms, pyelonephritis is probably due to haematogenous infection. Strains carrying K antigens are more commonly responsible for pyelonephritis, while most isolates from cystitis lack K antigens.

2. Diarrhoea: E. coli is the dominant member of the aerobic bacterial flora of the human intestine and is usually nonpathogenic in this location. But there are four groups of *E. coli* which can cause diarrhoeal disease, particularly in infants, and also in older children and adults. These have been called *enteropathogenic E. coli, enterotoxigenic E. coli, enteroinvasive E. coli* and *enterohaemorrhagic E. coli*.

Enteropathogenic *E. coli:* Attempts to implicate *E. coli* in the aetiology of diarrhoea began from 1885 when Escherich first isolated the bacterium from infantile diarrhoea. But it was only after serotyping schemes were developed that it became possible to establish a causal relationship between infantile diarrhoea and *E. coli* belonging

to some O antigen groups and carrying the B type of surface (K) antigen. These were called *enteropathogenic E. coli* (EPEC). At first only a few serotypes (0111:B4, 055:B5) were believed to be enteropathogenic, but the list has enlarged considerably and currently includes at least 25 serotypes. Examples of enteropathogenic serotypes are: 026:B6; 055:B5; 086:B7; 0111:B4; 0112:B11; 0114:B90; 0119:B14; 0125:B15; 0126:B16; 0127:B8; 0128:B12.

While diarrhoeal disease due to EPEC has been observed throughout the world, it has been particularly important in the developed countries. EPEC had caused several serious institutional outbreaks of diarrhoea in babies less than 18 months old, in Britain, Europe and North America from the late 1940s to the 1960s. From the 1970s, epidemic diarrhoea due to EPEC has become less frequent.

The pathogenic mechanism of EPEC has only recently been identified. They adhere tightly to enterocytes, leading to the loss of microvilli and cupping of enterocyte membranes to bacteria. They also produce a shigella-like toxin.

Enterotoxigenic *E. coli:* These are the strains that form heat labile or heat stable enterotoxins and produce diarrhoea. The importance of enterotoxigenic *E. coli* (ETEC) in diarrhoea has been established only in the 1970s. They are now known to be a major cause of diarrhoeal illness in children in the developing countries and the most important cause of 'travellers' diarrhoea'. (The name 'travellers' diarrhoea' is applied to diarrhoeal illness seen in persons from the developed countries within a few days of their visit to one of the developing countries.) ETEC sometimes cause a disease indistinguishable from cholera in infants ('cholera infantum'), young children and adults.

No biochemical markers are available to distinguish ETEC strains from other *E. coli*, so that their identification depends on the demonstration of the toxins. The methods available for the detection of the toxins are shown in Table 30.2. Because these are specialised procedures, only some laboratories are able to identify ETEC.

Most strains of ETEC belong to the O serogroups 6, 8, 15, 25, 27, 63, 78, 148 and 159. These are different from EPEC serotypes.

ETEC also possess surface properties, called 'colonisation factors', which promote their virulence. Colonisation factors may be pili or special types of protein K antigens.

Enteroinvasive *E. coli:* Some strains of *E. coli* isolated from stools of older children and adults with dysentery-like disease do not produce enterotoxins, but invade the intestinal epithelial cells as do dysentery bacilli. These have been called enteroinvasive *E. coli* (EIEC). They belong to a limited number of O serogroups: 28ac, 112ac, 124, 136, 143, 144, 152, 164. On instillation into the eyes of guinea pigs, EIEC cause keratoconjunctivitis (the Sereny test). This provides a diagnostic test for EIEC. The invasion of He La cells in tissue culture also provides another diagnostic method.

EIEC strains are often atypical biochemically. Many strains ferment lactose late or not at all, and may be anaerogenic. There is marked antigenic relationship between EIEC strains and shigellae. Because of their biochemical and antigenic resemblance to shigellae, many outbreaks due to EIEC have been mistaken for shigellosis.

Enterohaemorrhagic *E. coli:* This was identified only in 1983 following food borne outbreaks of haemorrhagic colitis caused by *E. coli* 0157: H7. It differs from the disease caused by EIEC in that fever is not always present, but the haemorrhage is marked. EHEC produces a cytotoxin which appears to be identical with *Shigella shiga* toxin. It has been called 'Verotoxin' because of its effect on vero cells in culture.

3. *Pyogenic infections: E. coli* may cause superficial infections such as wound infections and abscesses or deep infections such as peritonitis, cholecystitis and meningitis. *E. coli* is a common cause of meningitis in the newborn, but is much less so in older patients.

4. *Septicaemia: E. coli*, other coliforms and

TABLE 30.2

Methods for detection of ETEC enterotoxins

Assay	Heat labile toxin	Heat stable toxin
In vivo tests		
Ligated rabbit ileal loop		
Read at 6 hours	±	+
Read at 18 hours	+	−
Infant rabbit bowel	+	+
Infant mouse intragastric	−	+
Adult rabbit skin (vascular permeability factor)	+	−
In vitro tests		
Steroid production in Y1 mouse adrenal cell culture	+	−
Morphological changes in Chinese hamster ovary (CHO) cells.	+	−
Solid phase radioimmunoassay (RIA)	+	−
Enzyme-linked immunosorbent assay (ELISA)	+	−

Pseudomonas aeruginosa have replaced staphylococci as the commonest cause of septicaemia in many hospitals. This syndrome of Gram negative septicaemia consists of fever, hypotension and disseminated intravascular coagulation (endotoxic shock). The condition usually occurs in patients already debilitated. Mortality is very high.

Laboratory diagnosis

1. Diarrhoea: Laboratory diagnosis of *E. coli* diarrhoea has become increasingly complex following the recognition of the different mechanisms by which the bacillus can cause the disease. There is no single test which can identify all strains of *E. coli* responsible for diarrhoea. For detection of EPEC, fresh diarrhoeal stool is plated directly on blood agar and MacConkey's medium. After overnight incubation, the *E. coli* colonies are emulsified in saline on a slide and tested by agglutination with polyvalent and monovalent OB antisera against enteropathogenic serotypes. Growth on MacConkey agar is usually satisfactory for agglutination, but autoagglutination may occur rarely. Blood agar growth gives good results. The set of antisera used should cover all the enteropathogenic serotypes prevalent locally and hence the composition of the set will vary from place to place. As more than one serotype may be present in faecal plates, it is essential to test at least ten isolated colonies by agglutination. If all of them are negative, the confluent growth should be tested before the specimen is considered negative. The colony agglutinating with enteropathogenic serum should be further studied by biochemical tests to establish identity as *E. coli*.

If OB sera are not available, agglutination may be tested with O sera using heated bacterial suspensions. But as one O group may contain several OB serotypes, only some of which may be enteropathogenic, final identification requires the determination of the B type as well.

In looking for specific serotypes with distinctive properties, special indicator media may be employed. A modified MacConkey's medium in which lactose is replaced by sorbitol has been

employed for the detection of serotypes 0 55:B5 and 0111:B4 as these do not ferment sorbitol unlike most other *E. coli* strains. The fluorescent antibody technique has been used for the detection of specific serotypes of *E. coli* in faeces.

While the above procedures are necessary for the investigation of diarrhoeal outbreaks in infants and children, they are not recommended for the laboratory diagnosis of sporadic cases of diarrhoea. The aetiological significance of the isolation of EPEC from sporadic cases of diarrhoea is unclear and controversial. Agglutinating sera are costly and their routine use would be very expensive.

The identification of ETEC presents greater difficulties. It is easier to detect LT than ST. The rabbit ileal loop test is not practicable on a large scale, nor is it always dependable. Tissue culture tests (mouse adrenal cells or CHO cells) are more often employed. ELISA test is the simplest and is being increasingly used. As LT and cholera enterotoxin are antigenically similar, cholera antitoxin which is readily available can be used as the test reagent for ELISA. Detection of ST is done by the mouse intragastric test—inoculation into the stomach of newborn mice causing fluid accumulation.

The demonstration of EIEC is even more difficult. Till recently, the only method available was the Sereny test. The tissue culture test (He La cell) has now been standardised.

2. *Urinary tract infection:* Bacteriological diagnosis of urinary tract infection has undergone a marked change following the development by Kass of the concept of 'significant bacteriuria'. Normal urine is sterile, but during voiding may become contaminated with genital commensals. In order to avoid such contamination, urine used to be collected by catheterisation for culture. Any bacterial growth from catheterised urine was considered to denote infection. Even under ideal conditions, catheterisation leads to urinary infection in at least two per cent, and when precautions are inadequate, the risk is much higher. Hence catheterisation is no longer considered justifiable for diagnostic purposes. Instead, clean-voided, midstream samples of urine are employed for culture. Such specimens should be collected carefully to reduce contamination to the minimum. In the male, it is sufficient if midstream urine is collected after the prepuce is retracted and the glans penis cleaned with wet cotton. In the female, anogenital toilet is more important and should consist of careful cleaning with soap and water. Nonirritant antiseptics such as chlorhexidine have been recommended for vulval cleaning. Urine should be passed keeping the labia separated by fingers. The first portion of urine that flushes out commensal bacteria from the anterior urethra is discarded. The next portion of urine (midstream sample) is collected directly into a sterile wide mouthed container and transported to the laboratory without delay. Urine is a good medium for the growth of coliforms and other urinary pathogens, and hence delay in processing will vitiate the results of quantitative culture. If delay of more than 1–2 hours is unavoidable, the specimen should be refrigerated.

In quantitative cultures, midstream urine samples will give a biphasic distribution of colonies, most specimens containing either less than 10,000 or more than 100,000 bacteria per ml. Kass and other investigators have established that in the presence of active infection in the urinary tract, the urine will contain 100,000 bacteria or more per ml. This level is, therefore, considered to represent 'significant' bacteriuria. Counts of 10,000 bacteria or less per ml are due to contamination during voiding and are of no significance. Counts between the two levels are infrequent when the sample is collected properly and processed promptly. Such results should be considered equivocal and the culture repeated. Needless to say, interpretation of bacteriuria should always be with reference to the condition of the patient. In patients on antibacterial or diuretic drugs and with some bacteria like *Staph. aureus*, even low counts may be significant.

For quantitative culture, serial tenfold dilutions of urine are tested by the pour plate or surface culture methods. This, however, is too com-

plicated for routine diagnostic work, for which semiquantitative techniques are more convenient. The most widely used technique employs a 'standard loop' which transfers a fixed, small volume of urine. One loopful of urine is placed on a noninhibitory medium (blood agar) and another loopful on an indicator medium (MacConkey). The former medium gives a quantitative measurement of bacteriuria, while the latter enables a presumptive diagnosis of the bacterium. The isolates are identified by their properties.

Bacteriological investigation of urinary tract infection is not complete without an antibiotic sensitivity test of the isolate. *E. coli* and other common urinary pathogens develop drug resistance so frequently that no antibacterial therapy can be instituted meaningfully without testing individual strains. Resistance is often to multiple drugs and of the transferable variety. Antibiotic sensitivity tests may be done directly using the urine samples as inocula and the results confirmed by repeating the test with individual isolates

Because urinary tract infection is such a common problem and bacteriological facilities are not always available, several screening techniques have been introduced for the presumptive diagnosis of significant bacteriuria. These include the following: 1) Griess nitrite test — based on the absence of nitrite in normal urine. The presence of nitrite, detectable by a simple test, indicates the presence of nitrate reducing bacteria in urine; 2) catalase test — The presence of catalase as evidenced by frothing on addition of hydrogen peroxide indicates bacteriuria, though a positive result is obtained also in haematuria; 3) triphenyltetrazolium chloride (TTC) test — based on the production of a pink-red precipitate in the reagent caused by the respiratory activity of growing bacteria, 4) microscopic demonstration of bacteria in Gram stained films of urine; 5) glucose test paper — based on the utilisation of the minute amounts of glucose present in normal urine, by bacteria causing the infection; 6) dip slide culture methods — agar coated slides are immersed in urine or even exposed to the stream of urine during voiding, incubated and the growth estimated by colony counting or by colour change of indicators. None of the screening methods is as sensitive or as reliable as a culture.

The antibody coated bacteria test has been employed for localisation of the site of urinary infection. This is based on the assumption that bacteria coated with specific antibodies are present in the urine only when the kidneys are infected and not when the infection is confined to the bladder. Antibody coated bacteria are detected by immunofluorescence using fluorescent tagged antihuman globulin or by staphylococcal coagglutination.

3. Pyogenic infections: The specimens to be tested depend on the lesion. Cultures are made on MacConkey's medium, the isolate identified by biochemical methods and an antibiotic sensitivity test done as a guide to treatment.

4. Septicaemia: As for all septicaemias, diagnosis depends on the isolation of the bacterium by blood cuture. Antibiotic sensitivity tests are essential for choosing appropriate antibacterial therapy.

ALKALESCENS-DISPAR GROUP

A group of nonmotile bacilli, believed to be related to *Shigella* and associated with cases of dysentery were called the Alkalescens-Dispar (AD) group. *Sh. alkalescens* resembles *Sh. flexneri*, except in fermenting dulcitol and in producing alkali in litmus milk. *Sh. dispar* resembles *Sh. sonnei* in fermenting lactose, but is indole positive. An elaborate serotyping scheme was worked out for the identification and classification of the AD group but their antigens appear to be identical with those of *E. coli*. The AD group is now considered to be merely nonmotile, anaerogenic *E. coli* probably EIEC.

EDWARDSIELLA

This genus contains the single species *Edward-*

siella tarda which is a noncapsulated, motile bacillus with weak fermentative powers. The name *tarda* refers to its slow or weak fermentation of sugars. Of the sugars commonly used, only glucose and maltose are fermented. It forms indole and H_2S, utilises citrate and decarboxylates lysine and ornithine.

E. tarda is probably a normal intestinal inhabitant of snakes. It has been isolated from normal and diarrhoeaic human faeces. Its pathogenic role is uncertain, but it has been isolated from wounds, urine, blood, and from CSF in cases of fatal meningitis.

CITROBACTER

These are motile bacilli which utilise citrate, grow in KCN medium, produce H_2S and ferment lactose late or not at all. Two species are recognised, *Citro freundii*, which gives typical reactions and *Citro diversus*, which does not form H_2S.

Some strains (Ballerup-Bethesda group) exhibit extensive antigenic sharing with salmonellae. This may cause confusion in the diagnostic laboratory. Some strains (e.g., the Bhatnagar strain) have a Vi antigen serologically identical with the Vi antigen of *S. typhi* and *S. paratyphi C*. These may be used for estimation of Vi antibodies or for raising Vi antisera.

Citrobacter is a normal intestinal inhabitant. Many strains formerly called 'paracolons' belong to this group. It has been isolated from cases of enteric fever, but its aetiological role is not established. It may cause infections of the urinary tract, gall bladder, middle ear and meninges.

KLEBSIELLA

The genus *Klebsiella* consists of nonmotile, capsulated rods that grow well on ordinary media forming large, dome shaped, mucoid colonies of varying degrees of stickiness. Klebsiellae are widely distributed in nature, occurring both as commensals in intestines and as saprophytes in soil and water. They have been classified into three species based on biochemical reactions and into over 80 serotypes based on their capsular (K) antigens (Table 30.3).

K. pneumoniae

(Friedlander's bacillus, *Bacillus mucosus capsulatus*)

This bacillus was first isolated by Friedlander (1883) from fatal cases of pneumonia. It ferments sugars (glucose, lactose, sucrose, mannitol) with the production of acid and abundant gas. It is indole and MR negative and VP and citrate positive (IMViC −++). It forms urease. Strains formerly labelled as nonmotile *Aerobacter aerogenes (K. aerogenes)* are now considered to be *K. pneumoniae*. It is the second most populous member of the aerobic bacterial flora of the human intestines. It has become a very important cause of nosocomial infections, even replacing *E. coli* in some centres. It causes pneumonia, urinary infection, other pyogenic infections, septicaemia and, rarely, diarrhoea.

Klebsiella pneumonia is rare (0.5–13 per cent of cases in different series), but is a serious disease with a case fatality of about 80 per cent in the untreated. It occurs in middle-aged or older persons who have underlying medical problems such as alcoholism, chronic bronchopulmonary disease or diabetes mellitus. The disease is characterised by massive mucoid inflammatory exudate of lobar or lobular distribution, involving one or more lobes of the lung. Necrosis and abscess formation are more frequent than in pneumococcal pneumonia. Serotypes 1, 2 and 3 are usually responsible for pneumonia. Positive blood cultures can be obtained in 25 per cent of the cases.

K. pneumoniae is a frequent cause of urinary infection. As most strains are resistant to antibiotics, treatment poses serious problems. It also causes pyogenic infections such as abscesses, meningitis and septicaemia.

Some strains of *K. pneumoniae* isolated from cases of diarrhoea have been shown to produce an enterotoxin, very similar to the heat stable toxin of *E. coli*. The production of this toxin is determined by the presence of a plasmid.

TABLE 30 3
Differentiation of *Klebsiella* species

	K. pneumoniae	*K. ozaenae*	*K. rhinoscleromatis*
Gas from glucose	+	d	−
Acid from lactose	+	d	−
MR	−	+	+
VP	+	−	−
Citrate	+	d	−
urease	+	d	−
Malonate	+	−	+
Lysine	+	d	−

Diagnosis is made by culturing appropriate specimens and identifying the isolate by biochemical reactions Antibiotic sensitivity should invariably be done. Many strains carry plasmids determining multiple drug resistance.

K. ozaenae is a bacillus associated with ozaena, a disease characterised by foul smelling nasal discharge. Identification is difficult due to wide variation in the biochemical reactions of individual strains. *K. ozaenae* belongs to capsular types 3–6.

K. rhinoscleromatis: This bacillus causes rhinoscleroma, a chronic granulomatous hypertrophy of the nose prevalent in South Eastern Europe, India and Central America. The bacilli are seen intracellularly in lesions It can be identified by biochemical reactions and belongs to capsular type 3.

ENTEROBACTER

These are motile, capsulated, lactose fermenting bacilli which are indole and MR negative and VP and citrate positive. Two species are recognised, *E. cloacae* and *E. aerogenes* (Table 30.4).

E. cloacae is found in human and animal faeces, sewage, soil and water. It has also been found rarely in urine, pus and other pathological materials *E. aerogenes* includes strains formerly labelled as motile *Aerobacter aerogenes*. They are found in human and animal faeces, sewage, soil and water. They may cause urinary tract infections and hospital sepsis.

HAFNIA

This is a motile, nonlactose fermenting bacillus which is indole and MR negative and VP and citrate positive. Biochemical reactions are evident best at 22°C; at 37°C they may be negative or irregular. Only one genus is recognised, *H. alvei*. It is found in human and animal faeces, sewage, soil and water.

SERRATIA

This differs from *Hafnia* in forming a pink, red or magenta, nondiffusible pigment, prodigiosin. Only one species is of medical importance — *S. marcescens* (*B. prodigiosus*). It is a saprophyte found in water, soil and food. It may grow in sputum after collection and may suggest haemoptysis because of the pigment formed (pseudohaemoptysis) In recent years, human infections due to *S. marcescens* are being reported with increasing frequency, mainly in hospitalised patients. The bacillus has been associated with meningitis, endocarditis, septicaemia, peritonitis, respiratory infections and many other conditions. Multiple drug resistance is common in hospital strains

PROTEUS

Hauser (1885) isolated from faeces, water, sew-

TABLE 30.4
Differentiation between *E. cloacae* and *E. aerogenes*

	E. cloacae	*E. aerogenes*
Gas from glycerol	−	+
Aesculin hydrolysis	−	+
Lysine decarboxylase	−	+
Arginine dihydrolase	+	−

age and decomposing organic matter, motile Gram negative bacilli, characterised by 'swarming' growth on agar. They were named *Proteus* because of their pleomorphism, after the Greek god Proteus who could assume any shape. The genus *Proteus* is classified into five species based on biochemical differences (Table 30.5).

Morphology

They are Gram negative rods, 0.5µ x 1–3µ in size. Pleomorphism is frequent, with long filaments and granular forms. They possess peritrichate flagella and exhibit 'swarming' motility, best seen at 20°C. Neither capsules nor spores are formed. Many strains are fimbriate.

Cultural characteristics

They are aerobic and facultatively anaerobic. Good growth occurs on ordinary media. Cultures emit a characteristic putrefactive odour, variously described as 'fishy' or 'seminal'. Many strains produce 'swarming' on agar media. Discrete colonies can be seen only in very young cultures. Thereafter, the actively motile cells spread over the surface of the plate in successive waves, so as to form a thin filmy layer in concentric circles. Swarming is a striking feature of *Pr. vulgaris* and *Pr. mirabilis*, but does not occur with other species at 37°C, though it may be induced by growing on soft agar at 20°C–28°C. Swarming growth presents problems in the diagnostic laboratory when mixed growth is obtained in which *Proteus* is present with other bacteria. Several methods have been introduced to inhibit swarming. Increasing the concentration of agar (6 per cent) or incorporation of chloral hydrate (1:500), sodium azide (1:500), alcohol (5–6 per cent), sulphonamide, surface active agents or boric acid (1:1000) will inhibit swarming. Swarming does not occur on MacConkey's medium, on which smooth, colourless colonies are formed. The exact mechanism of swarming is not understood, but it is determined by motility. Non-motile strains do not swarm.

In broth, *Proteus* produces uniform turbidity with a slight, powdery deposit and an ammoniacal odour.

Biochemical reactions

The distinctive character of this genus is the deamination of phenylalanine to phenylpyruvic acid (PPA), which is always positive in *Proteus* and negative in all other members of the family Enterobacteriaceae. Hydrolysis of urea is another characteristic of the genus, but is negative in *Pr. inconstans (Providencia)*.

They are MR positive and VP negative. All ferment glucose producing acid and gas, excepting *Pr. rettgeri* and *Pr. inconstans* which are usually anaerogenic. Lactose is not fermented. Fermentation of sucrose, maltose and mannitol, liquefaction of gelatin, formation of indole and H_2S, citrate utilisation and ornithine decarboxylation form the basis of classification into different species.

Classification

Two species, *Pr. vulgaris* and *Pr. mirabilis*, were

described originally by Hauser. Morgan (1906) isolated motile bacilli from the stools of infants with summer diarrhoea. These form the prototype for the third species, *Pr. morganii*. The species *Pr. rettgeri* is named after Rettger, who first isolated it (1904). The status of the fifth species, *Pr. inconstans* is unsettled. This group was originally described by Stuart in Providence, U.S.A., and hence is widely known as the 'Providence group'. *Pr. inconstans* has been divided into two subgroups—one producing acid and gas in glucose and adonitol and the other forming acid only in glucose, but not in adonitol.

It has been held that these organisms should be classified into four separate genera, *Pr. vulgaris* and *Pr. mirabilis* which are very similar in their properties forming the genus *Proteus*, while *Pr. morganii*, *Pr. rettgeri* and *Pr. inconstans* constitute the genera *Morganella, Rettgerella* and *Providencia*, respectively.

Antigenic structure

The bacilli possess thermostable, somatic (O) antigens and thermolabile, flagellar (H) antigens, based upon which, several serotypes have been recognised within the different species. The antigenic scheme is common for *Pr. vulgaris* and *Pr. mirabilis*, but separate for the others. The ureases produced by *Pr. vulgaris, Pr. mirabilis* and *Pr. rettgeri* are antigenically indistinguishable, but that of *Pr. morganii* is distinct.

The H and O antigens of *Proteus* are of considerable historical interest. Weil and Felix (1916) studying *Proteus* bacilli, observed that flagellated strains growing on agar formed a thin surface film resembling the mist produced by breathing on glass and named this variety the 'Hauch' form (from Hauch, meaning 'film of breath'). Nonflagellated variants grew as isolated colonies without the surface film and were called 'Ohne Hauch' form (meaning 'without film of breath'). These names came to be abbreviated as the 'H' and 'O' forms. Subsequently, the terms H and O were extended to refer to the flagellar and somatic antigens of other bacilli as well. The terms are also used to designate the types of bacterial agglutination, the H type referring to the loose, fluffy masses formed when flagellated cells are clumped together, and the O type to the fine granular appearance of somatic agglutination.

Weil and Felix also observed that certain nonmotile strains of *Pr. vulgaris*, called the 'X strains', were agglutinated by sera from typhus fever patients. This heterophile agglutination due to the sharing of an alkali stable carbohydrate hapten by certain strains of *Proteus* and rickettsiae forms the basis of the Weil-Felix reac-

TABLE 30.5

Classification of the Genus Proteus

	Pr. vulgaris	*Pr. mirabilis*	*Pr. morganii*	*Pr. rettgeri*	*Pr. inconstans*
Gas from Glucose	+	+	+	d	d
Acid from Sucrose	+	d	−	d	d
Acid from Maltose	+	−	−	−	−
Acid from Mannitol	−	−	−	+	−
Gelatinase	+	+	−	−	−
Indole	+	−	+	+	+
H_2S	+	+	−	−	−
Citrate	d	d	−	+	+
Ornithine decarboxylase	−	+	+	−	−
Urease	+	+	+	+	−

tion for the diagnosis of some rickettsial infections. Three nonmotile strains are employed as the antigens for this agglutination test — *Pr. vulgaris* strains OX2, OX 19 and *Pr. mirabilis* OX K, which contain O antigen types 1, 2 and 3, respectively.

Pathogenicity

Proteus species are saprophytes, widely distributed in nature. They also occur as commensals in the intestine and sometimes on the skin over the moist parts of the body. They are opportunistic pathogens and may cause many types of infections, which may be acquired either naturally or in the hospital environment. Some studies indicate that the tribe Proteeae are responsible for approximately 10–15 per cent of nosocomial infections in the U.S.A. *Pr. mirabilis* accounts for the majority of *Proteus* infections in man. Several nosocomial infections by *Pr. rettgeri* and *Pr. inconstans* (Providentia) have been reported. *Pr. stuarti* (one of the subdivisions of *Pr. inconstans*) is emerging as an increasingly important cause of infection in burns.

Multiple drug resistant strains carrying transmissible *R* plasmids have become very important in hospital infection.

Urinary tract infection is the commonest disease caused by *Proteus*. Pyelonephritis due to *Proteus* is particularly toxic as the ammonia produced by the bacillus interferes with complement and other natural defence mechanisms. The alkalinity leads to necrosis of renal tubular epithelium, precipitation of magnesium phosphate and calculus formation.

Proteus strains may produce pyogenic lesions of various types, such as abscesses and infection of wounds, ear or respiratory tract. Both *Pr. morganii* and *Pr. inconstans* have been reported to cause infantile diarrhoea, but their aetiological role is in doubt. It has been observed that *Proteus* strains occur more abundantly in faeces during infection with other intestinal pathogens.

Laboratory diagnosis: Proteus can be readily isolated from urine or other sources. When *Proteus* infection is suspected, appropriate media (MacConkey, blood agar with 6% agar) should be employed to inhibit swarming so that any other bacteria present are not overgrown. Identification is by biochemical reactions. *Pr. mirabilis* is the most frequent species in clinical specimens. Antibiotic sensitivity testing should be done as drug resistance is very common. In contrast to other species, *Pr. mirabilis* is fairly uniform in its sensitivity to antibiotics and is usually susceptible to penicillin, ampicillin and cephalosporin. Many strains of *Pr. morganii* are sensitive to nitrofurantoin and tetracycline — drugs to which most other *Proteus* strains are resistant. Most strains of the different species are sensitive to kanamycin.

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31 Enterobacteriaceae — II Shigella

Dysentery is a clinical condition of multiple aetiology, characterised by the passage of loose motion mixed with blood and mucus. The two common types of dysentery are bacillary and amoebic. The causative agents of bacillary dysentery belong to the genus *Shigella*, so named after Shiga, who in 1896 isolated the first member of this genus from epidemic dysentery in Japan.

Morphology

Shigellae are short Gram negative rods, about 0.5 μ x 1–3 μ in size. They are nonmotile, nonsporing and noncapsulated. Fimbriae may be present.

Cultural characteristics

They are aerobes and facultative anaerobes, growing within a temperature range of 10°C–40°C, with an optimum temperature of 37°C and pH of 7.4. They grow on ordinary media, but less readily than other enterobacteria. After overnight incubation, colonies are small, about 2 mm in diameter, circular, convex, smooth and translucent. Occasionally on primary isolation and frequently in subcultures, a proportion of the colonies may be of the rough type. Colonies on MacConkey agar are colourless due to the absence of lactose fermentation. An exception is *Sh. sonnei* which ferments lactose late and forms pale pink colonies. Deoxycholate citrate agar (DCA) is a useful selective medium. Growth is inhibited on Wilson and Blair's bismuth sulphite medium.

Resistance

Shigellae are not specially resistant. They are killed at 56°C in one hour and by 1% phenol in 30 minutes. In water and ice they remain viable for 1–6 months. Boiling or chlorination of water and pasteurisation of milk destroy the bacilli. In the dark, dried on linen, they survive for 1–6 weeks. In faeces they die within a few hours due to the acidity produced by the growth of coliforms.

Sh. sonnei is, in general, more resistant than other species.

Shigellae are MR positive and reduce nitrates to nitrites. They cannot utilise citrate as the sole source of carbon, do not form H_2S and are inhibited by KCN. Catalase is produced, except by *Sh. dysenteriae* type 1. Glucose is fermented with the production of acid, without gas, except for the Newcastle and Manchester biotypes of *Sh. flexneri* type 6, which form gas. Fermentation of mannitol is of importance in classification and shigellae have traditionally been divided into mannitol fermenting and nonfermenting species. *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei* ferment mannitol, while *Sh. dysenteriae* does not. Exceptions are not infrequent. Lactose and sucrose are not fermented, except by *Sh. sonnei* which ferments them late. Adonitol, inositol and salicin are not fermented.

Antigenic structure: Shigellae possess one or more 'major' antigens and a large number of 'minor' somatic O antigens. Some strains possess K antigens. These are not relevant in typing, but

may sometimes interfere with agglutination by O antisera. Fimbrial antigens are also present. In general, the antigenic structure of shigellae is simple, compared to the complex structure of salmonellae. There is some degree of antigenic sharing between some members of the genus as well as between shigella and *E. coli*. Common fimbrial antigens may also occur, particularly in *Sh. flexneri*. It is, therefore, important that the identification of shigellae should be made by a combination of antigenic and biochemical properties and not by slide agglutination alone.

Classification

Shigellae are classified into four species or subgroups based on a combination of biochemical and serological characteristics. Serotypes are distinguished *within the species*. For epidemiological purpose, *Sh. sonnei* is classified into colicin types.

Sh. dysenteriae (subgroup A): This species of mannitol nonfermenting bacilli consists of ten serotypes. Type 1 is the bacillus originally described by Shiga *(Sh. shigae)*. It is indole negative and is the only member of the family that does not form catalase. It is unique among shigellae in forming exotoxins. Three types of toxic activity have been demonstrated in culture filtrates: (1) neurotoxin, which causes paralysis and death when injected into mice or rabbits, (2) enterotoxin which induces fluid accumulation in ligated rabbit ileal loop, and (3) cytotoxin which damages HeLa and monkey kidney cell cultures. It is not yet established whether these three activities are manifested by a single toxin or by separate toxins. The neurotoxin and enterotoxin are believed to be a single protein with a M.W. 55,000 to 60,000. It is heat labile and pronase sensitive. Shigella neurotoxin is the earliest exotoxin to have been detected in Gram negative bacilli. The cytotoxin may be a subunit of the larger toxin molecule.

Sh. dysenteriae type 1 toxin can be toxoided. The toxoid and antitoxin have been used for active immunisation and in treatment, without success.

Sh. dysenteriae type 1 causes the most severe type of bacillary dysentery and is most frequently associated with complications.

Sh. dysenteriae type 2 *(Sh. schmitzi)* forms indole and ferments sorbitol and rhamnose. Serotypes 3 – 7 were described by Large and Sachs in India and hence used to be known as the Large-Sachs group. Three further serotypes have since been described making a total of ten.

TABLE 31.1
Distinguishing features of Shigella species

Subgroup	A	B	C	D
Species	*Sh. dysenteriae*	*Sh. flexneri*	*Sh. boydii*	*Sh. sonnei*
Mannitol	–	*A*	*A*	*A*
Lactose	–	–	–	*A Late*
Sucrose	–	–	–	*A Late*
Dulcitol	–	–	*d*	–
Indole	*d*	*d*	*d*	–
Ornithine decarboxylase	–	–	–	+
Serotypes	*10*	*6+2 variants*	*18*	*(2 phases; 17 colicine types)*

A—Acid *d-Variable*

Sh. flexneri (Subgroup B): This group is named after Flexner, who described the first of the mannitol fermenting shigellae from Philippines (1900). This group is biochemically heterogeneous and antigenically the most complex among shigellae. Based on type specific and group specific antigens, they have been classified into six serotypes (1 – 6) and several subtypes (1a 1b;2a,2b;3a,3b,3c;4a,4b;5a,5b). In addition, two antigenic 'variants' called X and Y are recognised, which lack the type specific antigens. Serotype 6 is always indole negative and occurs in three biotypes, some of which form gas from sugars.

TABLE 31.2

Biotypes of *Sh. flexneri* Type 6

Biotype	Fermentation of	
	Glucose	Mannitol
Boyd 88	A	A
Manchester	AG	AG
Newcastle	A or AG	–

A—Acid AG—Acid and Gas

Sh. boydii (subgroup C): This group consists of dysentery bacilli that resemble *Sh. flexneri* biochemically, but not antigenically. The group is named after Boyd, who first described these strains from India (1931). Eighteen serotypes have been identified, the last three having been described only in 1985. *Sh. boydii* are isolated least frequently from cases of bacillary dysentery.

Sh. sonnei (subgroup D): This bacillus, first described by Sonne (1915) in Denmark, ferments lactose and sucrose late. It is indole negative. It is antigenically distinct and homogeneous, but may occur in two forms — phase I and phase II — the latter forming colonies that are larger, flatter and more irregular. On subculture, phase I produces both types of colonies, but phase II is considered to be a loss variation. Organisms in this phase may be isolated from patients, but are more common in convalescents and carriers.

Sh. sonnei causes the mildest form of bacillary dysentery. In many cases the disease may only be a mild diarrhoea. But *Sh. sonnei* infection persists as the most common shigellosis in advanced countries. For epidemiological purposes, *Sh. sonnei* has been classified into 17 colicin types.

Pathogenicity: Shigellae cause bacillary dysentery. Infection occurs by ingestion. The minimum infective dose is low, as few as 10–100 bacilli being capable of initiating the disease. The bacilli infect the epithelial cells of the villi in the large intestine and multiply inside them, spreading laterally to involve adjacent cells and penetrating into the lamina propria. Inflammatory reaction develops with capillary thrombosis, leading to necrosis of patches of epithelium, which slough off, leaving behind transverse superficial ulcers. Bacteraemia may occur infrequently in severe infections, particularly in malnourished children.

Though *Sh. dysenteriae* type 1 possesses an exotoxin, it appears to be much less important in pathogenesis than the ability of the bacillus to penetrate and multiply in the colonic mucosa. Nontoxigenic mutants can still cause dysentery, but not the noninvasive ones.

Bacillary dysentery has a short incubation period (1–7 days, usually 48 hours). The onset and clinical course are variable and are largely determined by the virulence of the infecting strain. The main clinical features are frequent passage of loose, scanty faeces containing blood and mucus, along with griping pain and tenesmus. Fever and vomiting may or may not be present. Complications are most often seen in infection with *Sh. dysenteriae* type 1 and include arthritis, toxic neuritis, conjunctivitis, parotitis and, in children, intussusception. Haemolytic uraemic syndrome may occur as a rare complication in severe cases. The severity of the disease may vary from acute fulminating dysentery to mild diarrhoea. As the term bacillary dysentery refers only to the more severe cases the term 'shigellosis' has been employed to include the whole spectrum of disease caused by shigellae.

Man is the only natural host for shigellae. Cap-

tive monkeys have been found infected, but such infections may have been of human origin. Experimentally, dysentery can be produced only in monkeys. Intraperitoneal injection in mice produces fatal bacteraemia and instillation into the eyes of guinea pigs causes keratoconjunctivitis. Intraluminal fluid accumulation follows injection of virulent strains into ligated rabbit ileal loops.

Epidemiology

Epidemics of bacillary dysentery have always accompanied wars and influenced their outcome. In several campaigns, more men have died of dysentery than were killed in battle. Epidemics in civilian communities are associated with poverty and insanitation.

The only source of infection is man—cases or carriers. The modes of transmission may be as follows: 1) direct, through contaminated fingers—'hand to mouth' infection, 2) through fomites; such as door handles, water taps, lavatory seats, 3) through water, 4) through contaminated food or drink. Shigellosis, especially *Sh. sonnei* infection, may occur as food poisoning, and 5) through flies which may transmit the infection as mechanical vectors.

Shigellosis is worldwide in distribution, but, epidemiologically, there are a number of differences between the nature and extent of the infection in the affluent and in the poor countries. Where environmental sanitation is good, as in Britain, shigellosis is mainly seen in young children and in special situations like mental hospitals (asylum dysentery). *Sh. sonnei* is the predominant infecting agent. In the U.S.A., *Sh. sonnei* is the main type in the north, while *Sh. flexneri* is more common in the south. Carrier rates for *Sh. flexneri* were found to vary from 0.1 per cent in New York to 11 per cent in New Mexico. In countries where environmental sanitation is poor, endemic shigellosis is found in all age groups and is caused by all species. In India, *Sh. flexneri* has always been the predominant species, having formed 50–85 per cent of isolates in different series. *Sh. dysenteriae* (8–25 per cent) and *Sh. sonnei* (2–24 per cent) are the next common species. *Sh. boydii* (0–8 per cent) has been isolated least frequently.

The picture has changed in recent years. After a long period of quiescence, *Sh. dysenteriae* type 1 suddenly appeared in an extensive and virulent epidemic form in Central America in 1968. In 1973, a similar outbreak started in Bangladesh and later in Sri Lanka. Several localised outbreaks were observed in India from 1974, followed by extensive epidemics in Kerala from 1981 and West Bengal from 1983. The epidemic strains showed multiple drug resistance.

Laboratory diagnosis

Diagnosis is made by isolating the bacillus from faeces. The ideal specimen is a direct swab of an ulcer taken by sigmoidoscopic examination. Fresh faeces can also be used. They should be inoculated without delay or transported in a suitable medium such as Sachs' buffered glycerol saline, pH 7.0–7.4. Highly alkaline transport media used for vibrios are inhibitory for shigellae.

It is best to use, for inoculation, mucus flakes if they are present in the sample. MacConkey and DCA plates are inoculated. After overnight incubation at 37°C, the plates are inspected for non-lactose fermenting colonies, which are tested for motility and biochemical reactions. Any non-motile bacillus that is urease, citrate, H_2S and KCN negative should be further investigated by biochemical tests (Table 31.1). Identification is confirmed by slide agglutination with polyvalent and monovalent sera.

The fluorescent antibody technique has been employed for the direct identification of shigellae in faeces, but is complicated by antigenic cross reactions and nonspecific fluorescence. The demonstration of antibodies in sera is of no value in diagnosis.

Treatment

Uncomplicated shigellosis is a self-limited condi-

tion that usually recovers spontaneously in a day or two. But in acute cases, particularly in infants and young children, dehydration has to be corrected promptly. Oral rehydration is adequate in most cases.

Routine antibacterial treatment is not indicated in dysentery. There is no convincing evidence that antibiotics either hasten recovery or prevent the carrier state. The very wide prevalence in shigellae of R factors conferring resistance to multiple antibiotics renders antibiotic therapy futile. Most strains prevalent in India now are resistant to chloramphenicol, streptomycin, sulphonamides, and some to ampicillin also. They were initially sensitive to cotrimoxazole, but resistant strains appeared later. In very serious infections, nalidixic acid has been life saving. Indiscriminate antibiotic therapy in dysentery will only help to increase the problem of drug resistance in intestinal bacteria. Antibiotics should, therefore, be reserved for the severe, toxic cases.

Control

As bacillary dysentery is an exclusively human infection transmitted by the faecal-oral route, control consists essentially in improvement of environmental sanitation.

The value of vaccination in the prophylaxis of dysentery is still uncertain. Killed vaccines proved to be toxic and ineffective. Many types of live oral vaccines have been tested, including a streptomycin dependent strain, avirulant mutants and *Shigella-E.coli* hybrids. They were shown to produce varying degrees of type specific immunity. The existence of several serogroups and types would appear to preclude effective immunisation. But vaccination is feasible as bacillary dysentery in a region is predominantly caused by one or two serotypes only at any time. The vaccines are, however, in the experimental stage.

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32 Enterobacteriaceae — III Salmonella

The genus Salmonella consists of bacilli that parasitise the intestines of a large number of vertebrate species and which infect man, leading to enteric fever, gastroenteritis, septicaemia with or without focal suppuration, and the carrier state.

The most important member of the genus is *Salmonella typhi*, the causative agent of typhoid fever. The typhoid bacillus was first observed by Eberth (1880) in the mesenteric nodes and spleen of fatal cases of typhoid fever and was isolated by Gaffky (1884). It came to be known as the Eberth-Gaffky bacillus or *Eberthella typhi*. Salmon and Smith (1885) described a bacillus which was believed to cause hog cholera. This bacillus, now called *S. cholerae-suis*, was the first of a series of similar organisms to be isolated from animals and man — the genus *Salmonella*. It was subsequently realised that the typhoid bacillus also belonged to this group, in spite of minor biochemical differences, and it was redesignated *S. typhi*, the genus *Eberthella* having been abolished. Salmonellae currently comprise about 2000 serotypes or species, all of them potentially pathogenic. For practical purposes, they may be divided into two groups: 1) The enteric fever group, consisting of the typhoid and paratyphoid bacilli that are exclusively or primarily human parasites; and 2) the food poisoning group, which are essentially animal parasites, but which can also infect man, producing gastroenteritis, septicaemia or localised infections.

Morphology

Salmonellae are Gram negative rods, about 1–3μ x 0.5–8μ in size. They are motile with peritrichate flagella, except for one type, *S. gallinarum-pullorum*, which is always nonmotile. Nonmotile mutants of other types may sometimes be found. They do not form capsules or spores, but may possess fimbriae.

Cultural characteristics

Salmonellae are aerobic and facultatively anaerobic, growing readily on simple media over a range of pH 6–8 and temperature 15°C–41°C (optimum 37°C).

Colonies are large, 2–3 mm in diameter, circular, low convex and smooth. They are more translucent than coliform colonies. On MacConkey and deoxycholate citrate media, colonies are colourless due to absence of lactose fermentation. On Wilson and Blair bismuth sulphite medium, jet black colonies with a metallic sheen are formed due to production of H_2S. *S. paratyphi* A and other species that do not form H_2S produce green colonies.

Selenite F and tetrathionate broth are commonly employed as enrichment media.

Biochemical reactions

Salmonellae ferment glucose, mannitol and maltose forming acid and gas. An important exception is *S. typhi* which is anaerogenic. Lactose, sucrose and salicin are not fermented. Indole is not produced. They are MR positive, VP negative and citrate positive. *S. typhi* and a few other salmonellae do not grow in Simmons' citrate

medium as they need tryptophan as growth factor. Urea is not hydrolysed, H_2S is produced, except by *S. paratyphi* A, *S. cholerae-suis* and some other species.

The enteric fever group may be separated biochemically (Table 32.1).

Resistance

The bacilli are destroyed at 55°C in one hour or at 60°C in 15 minutes. Boiling or chlorination of water and pasteurisation of milk destroy the bacilli. In polluted water and soil, they survive for weeks and in ice for months. Cultures may be viable for years if prevented from drying. They are killed within five minutes by mercuric chloride (1:500) or 5% phenol.

Antigenic structure

Salmonellae possess the following antigens based on which they are classified and identified — 1) flagellar antigen H, 2) somatic antigen O, and 3) a surface antigen Vi, found in some species. *Several strains carry fimbriae.* Fimbrial antigens are not important in identification, but may cause confusion due to their nonspecific nature and widespread sharing amongst enterobacteria.

H antigen: This antigen present on the flagella is a heat labile protein. It is destroyed by boiling or by treatment with alcohol, but not by formaldehyde. When mixed with antisera, H suspensions agglutinate rapidly, producing large, loose, fluffy clumps. The H antigen is strongly immunogenic and induces antibody formation rapidly and in high titre following infection or immunisation. The flagellar antigen is of a dual nature, occurring in one of two phases.

O antigen: The somatic O antigen is a phospholipid protein polysaccharide complex which forms an integral part of the cell wall. It is identical with endotoxin. It can be extracted from the bacterial cell by treatment with tricholoracetic acid, as first shown by Boivin (and therefore called the Boivin antigen). Treatment with phenol splits off the protein moiety, removing the antigenicity, but retaining the toxicity of the complex.

The O antigen is unaffected by boiling, alcohol or weak acids. When mixed with antisera, O antigen suspensions form compact, chalky, granular clumps. O agglutination takes place more slowly and at a higher temperature optimum (50°C–55°C) than H agglutination (37°C). The O antigen is less immunogenic than the H antigen and the titre of O antibody induced after infection or immunisation is generally lower than that of the H antibody.

The O *antigen is not a single factor,* but a mosaic of two or more antigenic factors. Salmonellae are classified into a number of groups based on the presence of characteristic O antigens on the bacterial surface.

Vi antigen: Many strains of *S. typhi* fail to agglutinate with the O antiserum when freshly isolated. This is due to the presence of a surface antigen enveloping the O antigen. (Felix and Pitt who first described this antigen believed that it was related to virulence.) It is analogous to the K anti-

TABLE 32.1
Biochemical characters of typhoid and paratyphoid bacilli

	Glucose	Xylose	d-Tartrate	Mucate
S. typhi	A	d	A	d
S. paratyphi A	AG	–	–	–
S. paratyphi B	AG	AG	–	AG
S paratyphi C	AG	AG	AG	–

dublin

gens of coliforms. It is heat labile. Bacilli inagglutinable with O antiserum become agglutinable after boiling or heating at 60°C for one hour. It is also destroyed by N HCl and 0.05 N NaOH. It is unaffected by alcohol or 0.2% formol.

Originally observed in *S. typhi*, the Vi antigen with similar antigenic specificity is present in *S. paratyphi* C and *S. dublin*, as well as in certain strains of *Citrobacter* (the Ballerup-Bethesda group). The Vi antigen tends to be lost on serial subculture. It is related to mouse virulence as judged by production of lethal infection by intraperitoneal inoculation, but its role in natural human disease is not known. It may act by coating the bacterial surface and preventing the antibacterial and opsonic effect of the O antibody. In human volunteer experiments, strains possessing Vi antigen were found to cause clinical disease more consistently than those lacking the antigen.

The *Vi antigen is poorly immunogenic* and only low titres of antibody are produced following infection. No Vi antibody is induced by the phenolised vaccine, though low titres are produced by the alcoholised vaccine. Detection of Vi antibody is not helpful for diagnosis of cases and hence the Vi antigen is not employed in the Widal test. It has been stated that total absence of Vi antibody in a proven case of typhoid fever indicates poor prognosis. The antibody disappears early in convalescence. Its persistence indicates the development of the carrier state. The Vi antigen affords a method of epidemiological typing of *S. typhi* strains based on specific Vi bacteriophages.

Antigenic variations

The antigens of salmonellae undergo phenotypic and genotypic variations.

1. H→O variation: This variation is associated with the loss of flagella. When salmonellae are grown on agar containing phenol (1:800), flagella are inhibited. This change is phenotypic and temporary. Flagella reappear when the strain is subcultured on media without phenol. Rarely, salmonellae may lose flagella by mutation. A stable nonmotile mutant of *S. typhi* is the 901-O strain which is widely employed for the preparation of O-agglutinable bacterial suspensions. Generally, loss of flagella is not total and there occurs only a diminution in the number of flagella and the quantity of H antigen. Flagellated cells are found in small numbers in such cultures. To obtain a population of motile cells, rich in H antigen from such cultures, selection may be carried out by using the Craigie's tube. This consists of a wide tube containing soft agar (0.2%) in the centre of which is embedded a short, narrow tube open at both ends, in such a way that it projects above the agar. The strain is inoculated carefully into the inner tube. After incubation, subcultures withdrawn from the top of the agar outside the central tube will yield a population of motile cells (Fig. 32.1). Instead of the Craigie's tube, a *U-tube of soft agar may be employed*, inoculation being made into one limb and subculture taken from the other limb.

2. Phase variation: The flagellar antigens of most salmonellae occur in one of two phases, i.e., the flagella may possess one or the other of two sets

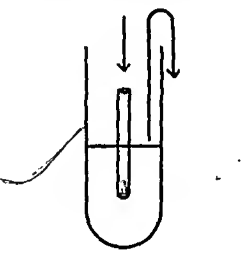

Fig. 32.1 Craigie's tube. Inoculation is made inside the inner tube and after incubation, subculture is taken from the surface of the medium in the outer tube.

of antigens. Phase 1 antigen is either specific for a species or shared by a few species only. Hence phase 1 is called the 'specific' phase. Phase 2 antigens are widely shared and hence it is called the 'nonspecific' or 'group' phase. Phase 1 antigens are designated a, b, c, d, etc. and after z, as z_1, z_2 etc. Phase 2 antigens are far fewer and are termed 1, 2, etc. In some species, antigens, belonging to phase 1 may occur as the phase 2 antigens (e.g., e, n, x, z_{15}). Strains that possess both phases are called diphasic. Some, like *S. typhi* occur in phase 1 only and are called monophasic.

A culture will contain cells with flagellar antigens of both phases, but generally one or the other phase will predominate so that the culture is agglutinated only by one of the phase antisera. For serotyping of salmonella isolates, it is necessary to identify the flagellar antigens of both phases. A culture in one phase may be converted to the other phase by passing through a Craigie's tube containing the homologous phase antiserum incorporated in the agar.

3. *V→W variation:* Fresh isolates of *S. typhi* generally carry a surface layer of Vi antigen that completely masks the O antigen. Such bacilli are agglutinable with the Vi antiserum but not with the O antiserum. This is called the V form. After a number of subcultures, the Vi antigen is completely lost. Such cultures are inagglutinable with Vi antiserum but readily agglutinable with O antiserum. This is called the W form. Intermediate stages during the loss of the Vi antigen, when the bacillus is agglutinable with both Vi and O antisera are called VW forms.

Other Vi-containing bacilli such as *S. paratyphi* C and *S. dublin* seldom have the O antigen completely masked by the Vi antigen.

4. *S→R variation:* The smooth-to-rough variation is associated with the change in the colony morphology and loss of the O antigen and of virulence. The colony becomes large, rough and irregular. Suspensions in saline are autoagglutinable. Conversion into R forms occurs by mutation. R forms may be common in laboratory strains maintained by serial subcultivation. S→R variation may be prevented to some extent by maintaining cultures on Dorset's egg media in the cold, or ideally by lyophilisation.

Mucoid colonies, associated with the development of a new mucoid or 'M' antigen have been described with *S. paratyphi* B and some other species.

5. *Variations in O antigen:* Changes in the structural formulae of O antigen may be induced by lysogenisation with some converting phages, resulting in the alteration of serotypes. Thus, *S. anatum* is converted into *S. newington* by one phage and the latter into *S. minneapolis* by another phage (Fig. 32.2).

It is likely that such changes taking place in nature contribute to the abundance of salmonella serotypes.

Classification

Salmonellae are classified broadly into four subgenera based on the biochemical reactions (Table 32.2).

S. anatum ———— serotype ———— 3,10: e,h: 1,6.

Lysogenisation ↓ with phage 15

S. newington ———— serotype ———— 3,15: e,h: 1,6.

Lysogenisation ↓ with phage 34

S. minneapolis ———— serotype ———— 3,15,34: e,h: 1,6.

Fig. 32.2 Phage conversion of Salmonella serotypes

TABLE 32.2

Biochemical reactions of Salmonella subgenera

Test	Subgenera I	II	III	IV
Lactose	–	–	+	–
Dulcitol	+	+	–	–
d-Tartrate	+	–	–	–
Malonate	–	+	+	–
Salicin	–	–	–	+
KCN	–	–	–	+

Subgenus I is the largest and most important, containing all the species that commonly cause human and animal infections. Other subgenera are of little importance in human disease. Subgenus II is common in reptiles. They have frequently been isolated from turtles and tortoises kept as pets. Subgenus III consists of bacilli formerly designated Arizona and later included in the genus Salmonella because of their antigenic similarity. Originally isolated from lizards, Arizona strains have since been obtained from reptiles, birds, domestic animals and man. They tend to be ignored in cultures as about 60 per cent of them ferment lactose promptly. Subgenus IV strains are but rarely encountered and they are best considered as typical members of subgenus II.

Serological classification of salmonellae is by the Kauffmann-White scheme.

Kauffmann-White scheme: This forms the basis of serotyping of salmonellae and depends on the identification by agglutination, of the structural formulae of O and H antigens of the strains. Salmonellae are initially classified into groups based on the presence of distinctive O antigen factors. [O antigen factors were formerly designated by Roman numerals (I, II, III, etc.), but are now indicated by Arabic numerals (1, 2, 3, etc.)]. The distinctive factor for group A is 2 and for group B, 4. So, any strain possessing factor 2 will be classified as group A and any strain possessing factor 4 as group B. The earlier groups are designated by capital letters, but the later groups are numbered, currently, upto group 65. Within each group, differentiation is by identification of phase 1 and phase 2 flagellar antigens (Table 32.3).

The Kauffmann-White Scheme gives species status to each serotype. On the other hand, Ewing considers that there are only three species of salmonellae, *S. cholera-suis*, *S. typhi* and

TABLE 32.3

Kaufmann-White Scheme: Illustrative examples

Group	Serotype	Antigen O	H Phase I	H Phase II
A	*S. paratyphi* A	1,2,12	a	–
B	*S. paratyphi* B	1,4,5,12	b	1,2
	S. typhimurium	1,4,5,12	i	1,2
–	*S. chester*	4,5,12	e,h	e,n,x
C_1	*S. paratyphi* C	6,7, (Vi)	c	1,5
	S. cholerae-suis	6,7	c	1,5
C_2	*S. muenchen*	6,8	d	1,2
D	*S. typhi*	9,12, (Vi)	d	–
	S. enteritidis	1,9,12	g,m	–
	S. gallinarum	1,9,12	–	–
E_1	*S. anatum*	3,10	e,h	1,6

S. enteritidis, all others being taken as serotypes of *S. enteritidis*. This suggestion has not found support.

Some 2000 serotypes of salmonellae have been identified so far. Most serotypes of medical importance fall into groups A to E. *Salmonella* species were named originally according to the disease caused by them (e.g., *S. typhi*), the animal source (e.g., *S. gallinarum*), after the persons who discovered them (e.g., *S. schottmulleri*), after the name of the person from whom the first strain was isolated (e.g., *S. thompsoni*) or after the place of isolation (e.g., *S. poona*). Due to the large profusion of serotypes, names are assigned only to new serotypes in subgenus I at present. New serotypes in other subgenera are to be designated only by their antigenic formulae.

Sometimes, more than one species may have the same antigenic formulae. Thus *S. gallinarum* and *S. pullorum* cannot be distinguished serologically, but they can be identified by biochemical reactions (*S. gallinarum* is anaerogenic and ferments dulcitol unlike *S. pullorum*). Important pathogens such as *S. typhi*, *S. paratyphi A* and *B*, and *S. typhimurium* can be further typed for epidemiological purposes by phage susceptibility, biochemical properties, bacteriocin production and antibiogram.

Pathogenicity. Salmonellae are strict parasites of animals or man. *S. typhi*, *S. paratyphi* A and usually, but not invariably, *S. paratyphi* B are confined to man. Other salmonellae are parasitic in various animals — domestic animals, rodents, reptiles — and birds. Some species exhibit host specificity — *S. abortus-equi* found only in horses, *S. abortus-ovis* in sheep and *S. gallinarum* in poultry. Others such as *S. typhimurium*, have a wide host range affecting animals, birds and man. Infection in animals may vary from an asymptomatic condition to fatal, and sometimes epizootic disease. *S. typhimurium* and *S. enteritidis* cause a fatal septicaemia in rats and mice. Preparations containing these bacilli ('rat virus') were once employed to eradicate rodents. Their use is dangerous and may lead to human infections. *S. pullorum* causes 'white diarrhoea' in chicks and *S. gallinarum* fowl typhoid.

Salmonellae cause the following clinical syndromes in man: 1) enteric fever, 2) septicaemia, with or without local suppurative lesions, and 3) gastroenteritis or food poisoning.

ENTERIC FEVER

The term enteric fever includes typhoid fever caused by *S. typhi* and paratyphoid fever caused by *S. paratyphi* A, B and C.

Typhoid fever was once prevalent all over the world and was not well demarcated from other prolonged fevers. A detailed study of the disease was presented by Bretonneau (1826) who identified the intestinal lesions. The name typhoid was given by Louis (1829) to distinguish it from typhus fever. Budd (1856) pointed out that the disease was transmitted through the excreta of patients. Eberth (1880) described the typhoid bacillus and Gaffky (1884) isolated it in pure culture. Its causative role was confirmed by Metchnikoff and Besredka (1900) by infecting apes experimentally. *S. paratyphi* A was isolated by Gwyn (1898) *S. paratyphi* B (*S. schottmulleri*) by Achard and Bensaude (1896) *S. paratyphi* C (*S. hirschfeldii*) by Uhlenhuth and Hubener (1908) from cases resembling typhoid fever.

The infection is acquired by ingestion. In human volunteer experiments, the ID50 was found to be 10 bacilli. It is possible that the bacilli may enter the body through the lymphoid tissues of the pharynx, but it is not known how frequent this mode of entry is. On reaching the gut, the bacilli attach themselves to the epithelial cells of the intestinal villi and penetrate to the lamina propria and submucosa. They are phagocytosed there by polymorphs and macrophages. The ability to resist intracellular killing and to multiply within these cells is a measure of their virulence. They enter the mesenteric lymph nodes, where they multiply and, via the thoracic duct, enter the bloodstream. A transient bacteraemia follows, during which the bacilli are seeded in the liver, gall bladder, spleen, bone marrow, lymph nodes,

Infective d

lungs and kidneys, where further multiplication takes place. Towards the end of the incubation period, there occurs a massive bacteraemia from these sites of multiplication, heralding the onset of clinical disease.

As bile is a good culture medium for the bacillus, it multiplies abundantly in the gall bladder and is discharged continuously into the intestine where it involves the Peyer's patches and lymphoid follicles of the ileum. These become inflamed, undergo necrosis and slough off, leaving behind the characteristic typhoid ulcers. Ulceration of the bowel leads to the two major complications of the disease — intestinal perforation and haemorrhage. During the 3–4 weeks that normally constitute the course of the disease, the intestinal lesions undergo healing.

The incubation period is usually 14 days, but may range from 5–20 days and appears to be related to the dose of infection. The clinical course may vary from a mild undifferentiated pyrexia (ambulant typhoid) to a rapidly fatal fulminating disease. The onset is usually gradual, with headache, malaise, anorexia, a coated tongue and abdominal discomfort with either constipation or diarrhoea. The typical features are a step-ladder pyrexia, with bradycardia, and toxaemia. A soft, palpable spleen is a constant finding. Hepatomegaly is also common. 'Rose spots' that fade on pressure appear on the skin during the second or third week, but are seldom noticeable in dark-skinned patients.

The most important complications are intestinal perforation, haemorrhage and circulatory collapse. Some degree of bronchitis or bronchopneumonia is always found. Some develop psychoses, deafness or meningitis. Cholecystitis, arthritis, abscesses, periostitis, nephritis, haemolytic anaemia, venous thromboses and peripheral neuritis are other complications found. Osteomyelitis is a rare sequel.

Convalescence is slow. In about 5–10 per cent of cases, relapse occurs during convalescene. The relapse rate is higher in patients treated with chloramphenicol (15–20 per cent).

S. paratyphi A and B cause paratyphoid fever which resembles typhoid fever, but is generally milder. *S. paratyphi* C may also cause paratyphoid fever, but more often it leads to a frank septicaemia with suppurative complications. Other salmonellae have on occasion been reported to cause enteric fever. These have included *S. dublin, S. barielly, S. sendai, S. enteritidis, S. typhimurium, S. eastbourne, S. saintpaul, S. oranienburg* and *S. panama*. Infection with *Alkaligenes faecalis* also may sometimes cause a similar clinical picture.

Epidemiology

Typhoid fever has been virtually eliminated from the advanced countries during the last several decades mainly as a result of improvements in water supply and sanitation, but it continues to be endemic in the poor nations of the world. The control of paratyphoid fever has not been so successful. The distribution of paratyphoid bacilli shows marked geographical differences. *S. paratyphi* A is prevalent in India and other Asian countries, Eastern Europe and South America, *S. paratyphi* B in Western Europe, Britain and North America; and *S. paratyphi* C in Eastern Europe and Guyana.

Enteric fever is endemic in all parts of India. Though there is no reliable information about its incidence in the country, typhoid fever has been estimated to affect 150–300 per 100,000 population. The proportion of typhoid to paratyphoid A is about 10:1. Paratyphoid B is rare and C very rare. The disease occurs at all ages, but is probably most common in the 5 – 20 year age group. The age incidence is related to the endemicity of the disease and the level of sanitation.

The source of infection is a patient, or far more frequently, a carrier. Patients who continue to shed typhoid bacilli in faeces for three weeks to three months after clinical cure are called convalescent carriers. Those who shed the bacilli for more than three months but less than a year are called temporary carriers and those who shed the bacilli for over a year are called chronic carriers. About

Ht, carrier

2–4 per cent of patients become chronic carriers. The development of carrier state is more common in females and in the older age groups (over 40 years). Some persons may become carriers following inapparent infection (symptomless excretor). The shedding of bacilli is usually intermittent. The bacilli persist in the gall bladder or kidney and are eliminated in the faeces (faecal carrier) or urine (urinary carrier), respectively. Urinary carriage is less frequent and is generally associated with some urinary lesion such as calculi or schistosomiasis.

Food handlers or cooks who become carriers are particularly dangerous. The best known of such typhoid carriers was Mary Mallon (Typhoid Mary), a New York cook, who, over a period of 15 years, caused at least seven outbreaks affecting over 200 persons.

Carriers occur with paratyphoid bacilli also. While *S. paratyphi* A occurs only in man, *S. paratyphi* B can infect animals such as dogs or cows, which may act as sources of human disease.

Typhoid fever occurs in two epidemiological types. The first is the endemic or residual typhoid that occurs throughout the year, though seasonal variations may sometimes be apparent. The second is the epidemic typhoid, which may occur in endemic or nonendemic areas. Typhoid epidemics are water, milk or food borne. Water borne epidemics, once so common, have become rare due to better control of drinking water. Milk borne epidemics also have become rare due to the general use of pasteurisation. Food borne outbreaks may be due to contamination of tinned foodstuffs, vegetables or shellfish which are eaten raw or undercooked. Some outbreaks of typhoid fever are still met with at times in the advanced countries (e.g., the water borne epidemic in Zermatt, Switzerland, in 1963; the food borne epidemic caused by infected corned beef in Aberdeen in 1964).

Laboratory diagnosis

Bacteriological diagnosis of enteric fever consists of the isolation of the bacilli from the patient and the demonstration of antibodies in his serum. A positive blood culture is diagnostic, while the same significance cannot be attached to isolation from faeces or urine. Demonstration of antibodies is not conclusive evidence of current infection. A third method, recently described, consists of the demonstration of typhoid bacilli antigen in blood or urine.

A. Blood culture

Bacteraemia occurs early in the disease and blood cultures are positive in approximately 90 per cent of cases in the first week of fever. The popular belief that blood culture for diagnosis of typhoid fever is useful only in the first week is erroneous. Blood culture is positive in approximately 75 per cent of cases in the second week, 60 per cent in the third week and 25 per cent thereafter till the subsidence of pyrexia. Blood cultures rapidly become negative on treatment with chloramphenicol.

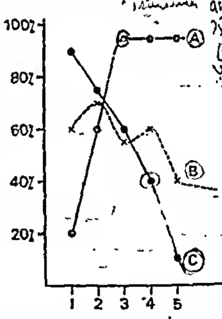

Fig. 32.3 Laboratory diagnosis of typhoid fever. The approximate percentages of tests found positive during different stages of the disease (from 1st to 5th week). A—Widal agglutination B—Faeces culture C—Blood culture.

About 5–10 ml of blood is collected by venepuncture and inoculated into a culture bottle containing 50–100 ml of 0.5 per cent bile broth. Blood contains substances that inhibit the growth of the bacilli and hence it is essential that the broth be taken in sufficient quantity to provide at least fourfold dilution of blood. The addition of liquoid (sodium polyanethol sulphonate) counteracts the bactericidal action of blood.

After incubation overnight at 37°C, the bile broth is subcultured on MacConkey agar. Pale nonlactose fermenting colonies that may appear on this medium are picked out for biochemical tests and motility. Salmonellae will be motile, indole and urease negative and fermenting glucose, mannitol and maltose, but not lactose or sucrose. The typhoid bacillus will be anaerogenic, while paratyphoid bacilli will form acid and gas from sugars. Identification of the isolate is by slide agglutination. A loopful of the growth from an agar slope is emulsified in two drops of saline on a slide. One emulsion acts as a control to show that the strain is not autoagglutinable. If *S. typhi* is suspected (i.e., when no gas is formed from glucose), a loopful of typhoid O antiserum (factor 9) is added to one drop of bacterial emulsion on the slide, and agglutination looked for after rocking the slide gently. Prompt agglutination indicates that the isolate belongs to Salmonella group D. Its identity as *S. typhi* is established by agglutination with the flagellar antiserum (anti-d serum). Quite often fresh isolates of *S. typhi* are in the V form and do not agglutinate with the O antiserum. Such strains may be tested for agglutination against anti-Vi serum. Alternatively, the growth is scraped off in a small amount of saline, boiled for 20 minutes and tested for agglutination with the O antiserum.

Where the isolate is a nontyphoid salmonella (producing gas from sugars), it is tested for agglutination with O and H antisera for groups A, B and C. For identification of unusual serotypes, the help of the National Salmonella Reference Centre should be sought. The National Salmonella Reference Centre in India is located at the Central Research Institute, Kasauli. The reference centre for salmonellae of animal origin is at the Indian Veterinary Research Institute, Izatnagar.

If salmonellae are not obtained from the first subculture from bile broth, subcultures should be repeated every other day till growth is obtained. Cultures should be declared negative only after incubation for ten days. To eliminate the risk of introducing contamination during repeated subcultures, and also for economy and safety, Castaneda's method of culture may be practised. In this, a double medium is used. The bottle of bile broth has an agar slant on one side. After inoculation of blood, the bottle is incubated in the upright position. For subculture, the bottle is merely tilted so that the broth runs over the surface of the agar. It is reincubated in the upright position. If salmonellae are present, colonies will appear on the slant.

An alternative to blood culture is the clot culture. Here 5 ml of blood is withdrawn from the patient into a sterile test tube and allowed to clot. The serum is pipetted off and used for the Widal test. The clot is broken up with a sterile glass rod and added to a bottle of bile broth. The incorporation of streptokinase (100 units per ml) in the broth facilitates lysis of the clot. Clot cultures yield a higher rate of isolation than blood cultures as the bactericidal action of the serum is obviated. Another advantage is that a sample of serum also becomes available. Even though in the early stages of the disease, agglutinins may be absent, a Widal test provides a baseline titre against which the results of tests performed later may be evaluated.

Faeces culture: Salmonellae are shed in the faeces throughout the course of the disease and even in convalescence, with varying frequency. Hence faecal cultures are almost as valuable as blood culture in diagnosis. A positive faecal culture, however, may occur in carriers as well as in patients. Isolation from faeces is successful from the early stage of the disease right upto convalescence when enrichment and selective media are used. Repeated sampling increases the rate of

isolation. Faecal culture is particularly valuable in patients on chloramphenicol as the drug does not eliminate the bacilli from the gut as rapidly as it does from the blood.

As salmonellae are greatly outnumbered in faeces by the normal flora, successful culture depends on the use of enrichment and selective media. Rectal swabs are not satisfactory. Faecal samples are plated directly on MacConkey, DCA and Wilson-Blair media. The last is highly selective and should be plated heavily. On MacConkey and DCA media, salmonellae appear as pale colonies. On Wilson-Blair medium, *S. typhi* forms large black colonies, with a metallic sheen. *S. paratyphi* A produces green colonies due to the absence of H_2S production.

For enrichment, specimens are inoculated into one tube each of selenite and tetrathionate broth, which are incubated for 12–18 hours before subculture onto plates.

Urine culture: Salmonellae are shed in the urine irregularly and infrequently. Hence urine culture is less useful than the culture of blood or faeces. Cultures are generally positive only in the second and third weeks and then only in about 25 per cent of cases. Repeated sampling improves the rate of isolation. Clean voided urine samples are centrifuged and the deposit inoculated into enrichment and selective media as for faecal culture.

Other materials for culture. Isolation may be obtained from several other sources, but they are not of much practical importance except in special situations. Bone marrow culture is positive in most cases. Culture of bile obtained by duodenal aspiration is usually positive and may be employed for the detection of carriers. Other materials which may yield isolation at times are rose spots, pus from suppurative lesions, CSF and sputum. At autopsy, cultures may be obtained from the gall bladder, liver, spleen and mesenteric lymph nodes.

B. Widal reaction

This is a test for the measurement of H and O agglutinins for typhoid and paratyphoid bacilli in the patient's sera. Two types of tubes are generally used for the test — a narrow tube with a conical bottom (Dreyer's agglutination tube) for the H agglutination, and a short round bottomed tube (Felix tube) for the O agglutination. Equal volumes (0.4 ml) of serial dilutions of the serum (from 1/10 to 1/640) and the H and O antigens are mixed in Dreyer's and Felix agglutination tubes, respectively, and incubated in a water bath at 37°C overnight. Some workers recommend incubation at 50°C–55°C. Control tubes containing the antigen and normal saline are set to check for autoagglutination. The agglutination titres of the serum are read. H agglutination leads to the formation of loose, cottonwoolly clumps, while O agglutination is seen as a disc-like pattern at the bottom of the tube. In both, the supernatant fluid is rendered clear.

The antigens used in the test are the H and O antigens of *S. typhi* and the H antigens of *S. paratyphi* A and B. The paratyphoid O antigens are not employed as they cross react with the typhoid O antigen due to the sharing by them of factor 12. The H agglutinable suspension is prepared by adding 0.1 per cent formalin to a 24-hour broth culture or saline suspension of an agar culture. For preparing the O suspension, the bacillus is cultured on phenol agar (1:800) and the growth scraped off in a small volume of saline. It is mixed with 20 times its volume of absolute alcohol, heated at 40°C–50°C for 30 minutes, centrifuged and the deposit resuspended in saline to the appropriate density. Chloroform may be added as a preservative. It is important to use standard smooth strains for antigen preparation. The strains used usually are the *S. typhi* 901 'O' and 'H' strains. Each batch of antigen should be compared with a standard.

The results of the Widal test should be interpreted taking into account the following:

1. The agglutinin titre will depend on the stage of the disease. Agglutinins usually appear by the end of the first week; so that blood taken earlier may give a negative result. The titre increases

steadily till the third or the fourth week, after which it declines gradually.

2. Demonstration of a rise in titre of antibodies, by testing two or more serum samples, is more meaningful than a single test. If the first sample is taken late in the disease, a rise may not be demonstrable. Instead, a fall in titre may be seen in some cases.

3. The results of a single test should be interpreted with caution. It is difficult to lay down levels of significance, though it is generally stated that titres of 1/100 or more for O agglutinins and 1/200 or more for H agglutinins are significant. It is necessary to obtain information on the distribution of agglutinin levels in 'normal sera' in different areas.

4. Agglutinins may be present on account of prior disease, inapparent infection or immunisation. H agglutinins persist longer than O agglutinins. Serum from an individual immunised with TAB vaccine will generally have antibodies to *S. typhi, S. paratyphi* A and B, while in case of infection, antibodies will be seen only against the infecting species.

5. Persons who have had prior infection or immunisation may develop an anamnestic response during an unrelated fever. This may be differentiated by repetition of the test after a week. The anamnestic response shows only a transient rise, while in enteric fever the rise is sustained.

6. Bacterial suspensions used as antigens should be free from fimbria. Otherwise false positive results may occur.

7. Cases treated early with chloramphenicol may show a poor agglutinin response.

The popularity of the Widal test in the diagnosis of enteric fever is undeserved, considering its fallacies and shortcomings.

C. Demonstration of circulating antigen

Typhoid bacillus antigens are consistently present in the blood in the early phase of the disease, and also in the urine of patients. The antigen can be demonstrated by the sensitised staphylococcal coagglutination test. *Staph. aureus* (Cowan I strain) which contains protein A, is stabilised with formaldehyde and coated with *S. typhi* antibody. When a 1% suspension of such sensitised staphylococcal cells is mixed on a slide with serum from patients in the first week of typhoid fever, the typhoid antigen present in the serum combines with the antibody attached to staphylococcal cells producing visible agglutination within two minutes. The test is rapid, sensitive and specific, but is not positive after the first week of the disease. Counterimmunoelectrophoresis and ELISA have also been used to detect typhoid antigen in blood and urine.

D. Other laboratory tests

1) A white cell count is useful. Leucopenia with a relative lymphocytosis is seen. Eosinophils are said to be absent, but in the tropics, with a high incidence of helminthic infestation, eosinophils are usually present. 2) Some workers have found the diazo test of urine very useful. Equal volumes of the patient's urine and the diazo reagent are mixed in a test tube and a few drops of 30% ammonium hydroxide added. On shaking the mixture, a froth develops, which is red or pink, if the test is positive. The diazo reagent consists of two stock solutions — solution A containing sulphanilic acid 0.5 g, Con. H_2SO_4 5 ml and distilled water 100 ml, and solution B containing sodium nitrite 0.5 g in distilled water 100 ml. For use, 40 parts of solution A are mixed with one part of solution B. The diazo reaction becomes positive usually between the 5th and 14th day of fever and becomes negative when the fever subsides. The test may be valuable where bacteriological facilities are not available.

Diagnosis of carriers

The detection of carriers is important for epidemiological and public health purposes. Laboratory tests are also useful in screening food handlers and cooks for carrier state.

The identification of faecal carriers is by isola-

TABLE 32.4

Bacteriophage types of S. typhi isolates from India tested in 1987-88 (Data from National Salmonella Phage Typing Centre, Lady Hardinge Medical College, New Delhi)

Phage type	*Percentages of predominant phage types among isolates from* North India	Central India	South India
A	31	46	51
E1	14	11	16
O	12	8	9
K1	5	1	6

Phage types sporadically isolated. 0 B1, C1, C4, C5, D1, D2, D6, E2, E14, F1, F2, F6, J1, K3, L1, M1, M3, 28, 38, 40, 42, 46

tion of the bacillus from faeces or from bile. The frequency and intensity of bacillary shedding vary widely and it is essential, therefore, to test repeated samples. Cholagogue purgatives increase the chance of isolation For the detection of urinary carriers, repeated urine cultures should be carried out.

The Widal reaction is of no value in the detection of carriers in endemic countries. The demonstration of Vi agglutinins has been claimed to indicate the carrier state While this is useful as a screening test, confirmation should be made by culture

The tracing of carriers in cities may be accomplished by the 'sewer swab' technique Gauze pads left in sewers and drains are cultured, and by tracing positive swabs, one may be led to the house harbouring a carrier Another technique of isolating salmonellae from sewage is filtration through millipore membranes and culturing the membranes on highly selective media such as those of Wilson and Blair.

Bacteriophage typing. Intraspecies classification of *S. typhi* for epidemiological purposes was made possible by bacteriophage typing, first developed by Craigie and Yen (1937). They found that a bacteriophage acting on the Vi antigen of the typhoid bacillus (Vi phage II) was highly adaptable The parent phage is called phage A. It could be made specific for a particular strain of typhoid bacillus by serial propagation in the strain Such adaptation was obtained by phenotypic or genotypic variation. At present, 97 Vi II phage types of *S. typhi* are recognised As phage typing of *S typhi* depends on the presence of Vi antigens, a proportion of strains (Vi negative) will be untypable. The phage type is stable. Apart from helping in tracing the source of epidemics, phage typing also provides information on the trends and patterns in the epidemiology of typhoid at the local, national and international levels. Phage typing is carried out at the National Phage Typing Centres and is coordinated by the International Reference Centre. The National Salmonella Phage Typing Centre for India is located at the Lady Hardinge Medical College, New Delhi. Table 32.4 shows the distribution of various phage types among typhoid bacilli isolated from different parts of India during 1987–1988 Phage types A and E1 are the most common and are present throughout India. However, the relative prevalence in different regions is subject to change from time to time.

The preponderance of one or two phages in a region limits the utility of phage typing as an epidemiological tool. Additional markers have, therefore, been employed for the subdivision of strains belonging to a phage type. These include 1) Nicolle's complementary phage typing of type A strains into 10 types, 2) Kristensen's biotyping based on fermentation of xylose and arabinose,

3) production of tetrathionate reductase, 4) bacteriocin production, and 5) antibiogram.

Phage typing has been applied also to *S. paratyphi* A and B, *S. typhimurium*, *S. enteritidis* and *S. dublin*. Among the *S. paratyphi* A isolates from India, phage types 1 and 2 are the most common (Table 32.5).

Prophylaxis: Typhoid fever can be effectively controlled by general measures, such as improvements in sanitation and provision of protected water supply.

Specific prophylaxis consists of vaccination, which was first popularised by Almroth Wright. The TAB vaccine which was in general use contained *S. typhi*, 1000 millions and *S. paratyphi* A and B, 750 millions each per ml, killed by heating at 50°C–60°C and preserved in 0.5 per cent phenol. The vaccine is given in two doses of 0.5 ml subcutaneously at an interval of 4–6 weeks. Local and general reactions lasting for one or two days are quite frequent. Such reactions may be avoided if the vaccine is administered in a dose of 0.1 ml intradermally. In nonendemic areas, vaccination is recommended for troops, medical and paramedical personnel. In endemic areas vaccination is recommended for all children, in whom a single dose might give adequate protection, which may be maintained for several years by the booster effect of repeated natural subclinical infections.

The use of polyvalent TAB vaccine was an accident of history. It was introduced in that form in World War I, as British troops had to serve in various parts of Europe, Africa and Asia where typhoid, paratyphoid A and B were endemic. No controlled field trial has been conducted with the TAB vaccine. In civilian practice, protection is mainly required against typhoid fever. If paratyphoid components are considered necessary either A or B may be added, but not both as only one of them is found in any one area. Therefore, in India, TAB vaccine has been replaced by the divalent typhoid-paratyphoid A vaccine, eliminating paratyphoid B which is very rare in the country.

The efficacy of phenolised typhoid vaccine has been established in controlled field trials. An alcoholised vaccine containing the Vi antigen fared poorly in field trials, though it gave good results in mouse protection tests. An acetone killed freeze dried vaccine was found to be effective in field trails. A recent (1986) field trial in Nepal using a single dose (0.25 μg) of Vi antigen gave 75 per cent protection. This holds promise.

Oral immunisation has been tried using killed vaccine as enteric coated tablets or live vaccine containing streptomycin-dependent strain or other mutants. Recently the *S. typhi* Ty 21a strain which is a stable mutant lacking the enzyme UDP-galactose-4-epimerase (Gal E mutant) has been used as a live vaccine. On ingestion it initiates

TABLE 32.5

Bacteriophage types of *S. paratyphi* A isolates from India tested in 1987-88
(Data from National Salmonella Phage Typing Centre, Lady Hardinge Medical College, New Delhi).

Phage type	*Percentage of phage types among isolates from* North India	Central India	South India
1	51	64	68
2	14	26	18
4	1	1	2
6	3	3	5

infection, but 'self-destructs' after four or five cell divisions, and therefore cannot induce any illness. A large field trial with the vaccine in Egypt gave very encouraging results, but subsequent experience has not been as satisfactory.

Typhoid bacilli are primarily intracellular parasites and cell mediated immunity rather than humoral antibodies may be more relevant in protection against the disease. Cell mediated immunity develops during the course of the disease. Cellular immunity to the typhoid bacillus is common in populations in endemic areas. Absence of CMI has been claimed to indicate susceptibility. The killed vaccine currently used does not stimulate CMI.

Treatment: Specific antibacterial therapy for enteric fever became available only in 1948 with the introduction of chloramphenicol. Though *S. typhi* is susceptible *in vitro* to many antibiotics such as streptomycin and tetracycline, they are ineffective *in vivo*. Ampicillin, amoxycillin, furozolidine, and cotrimoxazole have been found useful in the treatment of typhoid fever.

While antibacterial therapy has been so effective in the treatment of cases, it has been disappointing in the treatment of carriers. A combination of antibacterial therapy along with the vaccine has been tried in the eradication of carrier state. This combination has also been used to prevent relapses. Elimination of the carrier state may require heroic measures such as cholecystectomy, pyelolithotomy or nephrectomy.

Drug resistance: Though occasional resistant strains had been identified in the laboratory, resistance to chloramphenicol did not pose any problem in typhoid fever till 1972, when resistant strains emerged in Mexico and in Kerala (India). In Mexico, the resistant strain caused an explosive epidemic, with high mortality. Travellers who got infected in Mexico had, on occasion, conveyed the resistant strain to North America and Europe, but it did not get established in these areas. Chloramphenicol resistant typhoid fever has become a problem in many countreis in South East Asia.

In India, chloramphenicol resistant typhoid fever appeared in epidemic form first in Calicut (Kerala) in early 1972. It became endemic and was confined to Kerala till 1978. Subsequently resistant strains appeared in many other parts of India, though the majority of *S. typhi* isolates continued to be sensitive. In India, resistance was originally confined to phage type DIN, but later it appeared in types C5, A, O and others. Resistance has been due to a transmissible plasmid carrying *R* determinants to chloramphenicol, streptomycin, sulphonamide and tetracycline. Plasmid borne drug resistance has been seen very rarely in *S. paratyphi* also

Salmonella gastroenteritis

Salmonella gastroenteritis or food poisoning is generally a zoonotic disease, the source of infection being animal products. It may be caused by any salmonella except *S. typhi*. The first instance of salmonella food poisoning to have been identified was in 1888, when Gaertner in Germany isolated a bacillus (*S. enteritidis*) from the meat of an emergency-slaughtered cow and from the cadaver of a fatal case of food poisoning caused by the meat. In 1898, Durham in England and de Nobele in Belgium isolated *S. typhimurium* from meat and from food poisoning cases. A very large number of salmonellae have since been identified from cases of gastroenteritis and food poisoning, but a few species account for the majority of cases. In most parts of the world, *S. typhimurium* is the commonest species. Some other common species have been *S. haldar, S. enteritidis, S. heidelberg, S. agona, S. virchow, S. seftenberg, S. indiana, S. newport* and *S. anatum*.

Human infection results from the ingestion of contaminated food. The most frequent sources of salmonella food poisoning are poultry, meat, milk, cream and eggs. Human carriers do occur, but their role is minimal when considered in relation to the magnitude of infection from animals. Food contamination may also result from droppings of rats, lizards or other small animals. Gas-

troenteritis may occur without food poisoning as in cross infection in hospitals.

Clinically, the disease develops after a short incubation period of 24 hours or less, with diarrhoea, vomiting, abdominal pain and fever. It may vary in severity from the passage of one or two loose stools to an acute cholera-like disease. It usually subsides in 2–4 days, but in some, a more prolonged enteritis develops, with passage of mucus and pus in faeces, resembling dysentery. In a few, typhoidal or septicaemia type of fever may develop.

Laboratory diagnosis is made by isolating the salmonella from the faeces. In outbreaks of food poisoning, the causative article of food can often be known by taking a proper history. Isolation of salmonellae from the article of food confirms the diagnosis.

Control of salmonella food poisoning requires the prevention of food contamination Food may become contaminated at various levels, from natural infection in the animal or bird, to contamination of the prepared food Proper cooking of food destroys salmonellae.

Treatment of uncomplicated, noninvasive salmonellosis is symptomatic. Antibiotics should not be used. Not only do they not hasten recovery, but they may actually increase the period of faecal shedding of the bacilli.

Salmonella septicaemia

Certain salmonellae, *S. cholera suis* in particular, may cause septicaemic disease with focal suppurative lesion, such as osteomyelitis, deep abscesses, endocarditis, pneumonia and meningitis. Antecedent gastroenteritis may or may not be present. The case fatality may be as high as 25 per cent.

Salmonellae may be isolated from the blood or from the pus from the suppurative lesion. Faeces culture may also sometimes be positive. Septicaemic salmonellosis should be treated with chloramphenicol or other appropriate antibiotics as determined by sensitivity tests.

Multi-resistant salmonellae

R factors conferring multiple drug resistance have become widely disseminated among salmonellae. The clinical significance of this phenomenon was first observed during the studies of human and veterinary infections with drug resistant *S. typhimurium* phage type 29 in England in the 1960's. Human infections were initially gastroenteritis due to spread from infected animals, through food. Subsequently, some salmonellae appear to have changed their ecology in some ways From being responsible for zoonotic infections only, as formerly, some multi-resistant salmonellae have now become important agents of hospital cross infections. Such nosocomial salmonellosis manifests particularly in neonates as septicaemia, meningitis and suppurative lesions. Diarrhoea may not always be present.

In India, several hospital outbreaks of neonatal septicaemia caused by multi-resistant salmonellae have occurred in recent years, as for example, the outbreak caused by *S. alachua* in Calcutta from 1972, *S. newport* in Delhi during 1975–76, *S. bareilly* in Chandigarh in 1978 and the protracted outbreaks caused by *S. typhimurium* in different parts of the country from 1978. Mortality in neonates is very high unless early treatment is started with antibiotics to which the infecting strains is sensitive.

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33 Vibrio

Vibrios are Gram negative, rigid, curved rods that are actively motile by means of a polar flagellum. The name 'vibrio' is derived from the characteristic vibratory motility (from—vibrare, meaning to vibrate). They are asporogenous and noncapsulated. Vibrios are present in marine environments and surface waters worldwide. The most important member of the genus is *Vibrio cholerae*, the causative agent of cholera. It was first isolated by Koch (1883) from cholera patients in Egypt, though it had been observed earlier by Pacini (1854) and others.

Vibrio cholerae

Morphology: The cholera vibrio is a short, curved, cylindrical rod, about 1.5µ x 0.2—0.4µ in size, with rounded or slightly pointed ends. The cell is typically comma shaped (hence the name *V. comma*), but the curvature is often lost *on subculture. S shaped or spiral forms may be* seen due to two or more cells lying end to end. Pleomorphism is frequent in old cultures. In stained films of mucous flakes from acute cholera cases, the vibrios are seen arranged in parallel rows, described by Koch as the 'fish in stream' appearance. It is actively motile, with a single polar flagellum. The motility is of the darting type, and when acute cholera stool or a young culture is examined under the microscope, the actively motile vibrios suggest a 'swarm of gnats' The vibrios stain readily with aniline dyes and are Gram negative and nonacid fast (Fig. 33.1)

Cultural characteristics: The cholera vibrio is strongly aerobic, growth being scanty and slow anaerobically. It grows within a temperature range of 16°C–40°C (optimum 37°C). Growth is better in an alkaline medium the range of pH being 6.4–9.6 (optimum 8.2). Growth is inhibited by 7% NaCl.

It grows well on ordinary media. On nutrient agar, after overnight growth, colonies are moist, translucent, round disks, about 1–2 mm in diameter, with a bluish tinge in transmitted light. The growth has a distinctive odour. On MacConkey's agar, the colonies are colourless at first, but become reddish on prolonged incubation due to the late fermentation of lactose. On blood agar, colonies are initially surrounded by a zone of greening, which later becomes clear due to

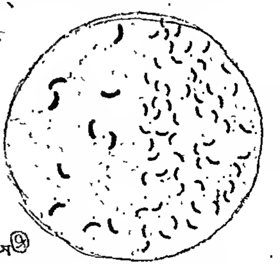


Fig. 33.1 Cholera vibrios Right—Gram stain Left—Flagellar stain showing single polar flagellum

haemodigestion. In gelatin stab culture, infundibuliform or napiform liquefaction occurs in three days at 22°C. In peptone water, growth occurs in about six hours as a fine surface pellicle, which breaks up on shaking into membranous pieces. Turbidity and a powdery deposit develop on continued incubation.

A number of special media have been employed for the cultivation of cholera vibrios. They may be classified as follows:

A. Holding or transport media: 1. Venkatraman-Ramakrishnan (VR) medium: A simple modified form of this medium is prepared by dissolving 20 G crude sea salt and 5 G peptone in one litre of distilled water and adjusting the pH to 8.6–8.8. It is dispensed in screwcapped bottles in 10–15 ml amounts. About 1–3 ml stool is to be added to each bottle. In this medium vibrios do not multiply, but remain viable for several weeks.

2. Cary-Blair medium: This is a buffered solution of sodium chloride, sodium thioglycollate, disodium phosphate and calcium chloride at pH 8.4. It is a suitable transport medium for salmonella and shigella as well as for vibrios.

B. Enrichment media: 1) Alkaline peptone water at pH 8.6; 2) Monsur's taurocholate tellurite peptone water, pH 9.2. Both these are good transport as well as enrichment media.

C. Plating media: The classical selective media for vibrios, Dieudonne's (1909) blood alkali agar and Aronson's (1915) sucrose-dextrin agar, are no longer in routine use now.

1. Alkaline bile salt agar (BSA) pH 8.2: This simple medium has stood the test of time and is still widely used. The colonies are similar to those on nutrient agar.

2 Monsur's gelatin taurocholate trypticase tellurite agar (GTTA) medium: Cholera vibrios produce small, translucent colonies with a greyish black centre and a turbid halo. The colonies become 3–4 mm in size in 48 hours.

3. TCBS medium: This medium containing thiosulphate, citrate, bromthymol blue and sucrose is available commercially and is very widely used at present. Cholera vibrios produce large yellow convex colonies which may become green on continued incubation.

Vibrio colonies may be identified by the 'string test'. A loopful of the growth is mixed with a drop of 0.5% sodium deoxycholate in saline on a slide. If the test is positive, the suspension loses its turbidity, becomes mucoid and forms a 'string' when the loop is drawn slowly away from the suspension.

Biochemical reactions: Carbohydrate metabolism is fermentative, producing acid, but no gas. Cholera vibrios ferment glucose, mannitol, maltose, mannose and sucrose, but not inositol, arabinose, or lactose, though lactose may be split very slowly. Indole is formed and nitrates are reduced to nitrites. These two properties contribute to the 'cholera red reaction' which is tested by adding a few drops of sulphuric acid to a 24-hour peptone water culture. With cholera vibrios, a reddish pink colour is developed due to the formation of nitroso indole. Catalase and oxidase tests are positive. Methyl red and urease tests are negative. Vibrios decarboxylate lysine and ornithine, but do not utilise arginine. Gelatin is liquefied. Vibrios elaborate several enzymes including collagenase, elastase, nucleotidase, decarboxylase, lipase, mucinase and neuraminidase (receptor destroying enzyme).

Two biochemical reactions of importance in the classification of vibrios are the Voges-Proskauer test and haemolytic activity. The classical cholera vibrio is V.P. negative and nonhaemolytic, while the el Tor vibrio, as originally described is V.P. positive and haemolytic. The haemolysis test has been modified several times in an attempt to obtain uniform results. As recommended by Greig (1914), the test was done by incubating a 72-hour broth culture of the test strain with 5% washed goat erythrocytes at 37°C for two hours and looking for haemolysis after storing overnight in the ice chest. As modified by Feeley and Pittman (1963), equal volumes (0.5 ml) of 24-hour heart infusion broth culture and 1%

sheep erythrocyte suspension are mixed, incubated at 37°C for two hours and examined for haemolysis after holding at 4°C overnight. More consistent results are obtained when 1% glycerol is added to the broth as a stabiliser. Modifications have also been proposed in which haemolysis is tested by the plate method.

Resistance: Cholera vibrios are susceptible to heat, drying and acids. It is destroyed at 55°C in 15 minutes. Dried on linen or thread, it survives for 1–3 days, but on coverslips, it dies in about three hours. Survival in water is influenced by its pH, temperature, presence of organic pollution and other factors. In general, the el Tor vibrio survives longer than the classical cholera vibrio. In shallow well water at pH 7.6 – 8.8, their average survival periods were 19.8 and 7.5 days, respectively. In the laboratory, vibrios survive for months in sterile sea water, and this has been suggested as a method for the survival of vibrios in nature. In grossly contaminated water, such as the Ganges water in India, the vibrios do not survive for any length of time, due to the apparently large amounts of vibriophages present. They survive in clean tap water for thirty days. In untreated night soil, they may survive for several days. Vibrios are suceptible to the common disinfectants. The destruction of vibrios by chlorine in water is influenced by the extent of organic pollution.

On fruits, they survive for 1–5 days at room temperature and for a week in the refrigerator. In general, food materials left at room temperature do not act as important sources of infection for longer than a day or two, but those stored in the cold may harbour vibrios for more than two weeks.

They are killed in a few minutes in gastric juice of normal acidity but they may survive for 24 hours in achlorhydric gastric juice.

Classification: In the past, many oxidase positive, motile, curved rods had been rather loosely grouped as vibrios. Precise criteria have recently been suggested for differentiating vibrios from closely related genera (Table 33.1). Heiberg (1934) classified vibrios into six groups based on the fermentation of mannose, sucrose and arabinose. Two more groups have been added subsequently. Cholera vibrios belong to Group I (Table 33.2).

The primary purpose of classifying vibrios is to distinguish between pathogenic and nonpathogenic vibrios and the major problem is the identification, definition and classification of the el Tor and the so-called NAG vibrios in relation to the classical cholera vibrios.

The el Tor vibrios

Gotschlich (1905) isolated a vibrio from six Haj

TABLE 33.1

Differentiation of vibrios from allied genera

Genus	Oxidation-Fermentation (Hugh-Leifson Test)		Utilisation of amino-acids			String test
	Oxidation	Fermentation	Lysine	Arginine	Ornithine	
Vibrio	+	+1	+	–	+	+
Aeromonas	+	+2	–	+	–	V
Pseudomonas	+	–	V	V	V	–
Plesiomonas	+	+	+	+	+	–

Note : 1 = no gas produced; 2 = gas may or may not produced;
V = *reaction variable.*

TABLE 33.2

Heiberg grouping of vibrios

Group	Fermentation of mannose	Sucrose	Arabinose
I	A	A	–
II	–	A	–
III	A	A	A
IV	–	A	A
V	A	–	–
VI	–	–	–
VII	A	–	A
VIII	–	–	A

pilgrims who had died of dysentery or gangrene of the colon at the Tor quarantine station on the Sinai Peninsula. This was called the el Tor vibrio. It was identical to cholera vibrios in all respects except that it was V.P. positive and haemolytic. As the el Tor vibrios were subsequently isolated from normal human intestines and water samples, they were considered to be nonpathogenic. In 1937, el Tor vibrios were isolated from an outbreak of choleraic disease in Sulawesi (Celebes, Indonesia). Subsequently, el Tor vibrios were repeatedly found in Sulawesi, where they were responsible for an endemic cholera-like disease. But outside this endemic area, el Tor vibrios continued to be considered nonpathogenic. Besides the V.P. and haemolysis tests, several other criteria were proposed for differentiation between the el Tor and classical cholera vibrios. These have included Tanamal's soda serum agglutination and flocculation tests, Wahba and Takla's copper sulphate flocculation, Gispen's heat agglutination, Mayer's chloroform agglutination tests, Gan and Tjia's trypsin test, Tomisawa's aldehyde test and others.

In 1961, the problem of differentiation between classical cholera and el Tor vibrios assumed great practical importance following the spread of pathogenic el Tor strains from Sulawesi to other parts of South East Asia. This marked the beginning of the seventh pandemic of cholera, which has yet to subside, being active even now in many areas. The strains isolated early in the pandemic were haemolytic vibrios, but from late 1962, the majority of the isolates has been nonhaemolytic. Due to the inconstant results in haemolysis and other criteria for distinguishing between el Tor and classical cholera vibrios, new methods had to be developed. The following criteria are used at present (Table 33.3).

TABLE 33.3

Differentiation between classical cholera and el Tor vibrios

Test	Classical cholera	el Tor
Haemolysis	–	+ @
Voges-Proskauer	–	+ @
Chick erythrocyte agglutination	–	+
Polymyxin B sensitivity	+	–
Group IV phage susceptibility	+	–

Note : @ — Strains isolated after 1961 give variable results.

1. *Chick red cell agglutination test:* A loopful from an agar culture is emulsified in a drop of saline on a slide and a drop of 2.5% chick erythrocyte suspension added. Clumping of the erythrocytes within a minute denotes a positive test. All el Tor strains are positive and classical cholera vibrios negative.

2. *Sensitivity to polymyxin* B: This is tested by the disc diffusion method using discs containing 50 units of the antibiotic. All strains of classical cholera vibrio are sensitive to polymyxin at this concentration and all strains of el Tor vibrio resistant. Occasional exceptions have been observed.

3. *Sensitivity to cholera phage group* IV: All strains of classical cholera vibrio are lysed by Mukerjee's group IV phage at routine test dilution, while all el Tor strains are insusceptible. This is considered to be the most dependable test now available for differentiating between el Tor and classical strains. Differentiation between them is no longer important in laboratory diagnosis of cholera as both can produce clinical ill-

TABLE 33.4

Antigenic classification of vibrios according to Gardner and Venkatraman

VIBRIO

- Group A. Cholera and cholera-like vibrios.
  General biochemical similarity
  Common H antigen
  - O subgroup I
    - nonhaemolytic cholera vibrios
      - Ogawa
      - Inaba
      - Hikojima
    - Haemolytic el Tor vibrios VPH
      - Ogawa
      - Inaba
      - Hikojima
  - O subgroups II, III, IV, V and VI individual races.
    Mostly haemolytic. (These are called nonagglutinable vibrios.) NAG NAV (NCV)
- Group B. Unrelated to cholera vibrios, biochemically and antigenically

ness, but the distinction is of epidemiological significance.

Nonagglutinable (NAG) vibrios

Gardner and Venkataraman (1935–36) presented an antigenic classification of vibrios (Table 33.4). They were divided into two large groups, Group A consisting of cholera vibrios and biochemically similar vibrios possessing a common H antigen, and Group B a heterogeneous collection of vibrios distinct from Group A biochemically and antigenically.

Group A vibrios were divided into six subgroups, I to VI, based on the O antigens. Vibrios belonging to O subgroup I were further divided by the Greig test into the nonhaemolytic cholera vibrios and the haemolytic el Tor vibrios, both of which were antigenically indistinguishable. Based on differences in minor O antigens, both haemolytic and nonhaemolytic O subgroup I vibrios were divided into three serotypes — Ogawa, Inaba and Hikojima (Table 33.5).

TABLE 33.5

O serotypes of cholera vibrios

Serotype	*O antigens*
Ogawa	AB
Inaba	AC
Hikojima	ABC

Vibrios resembling *V. cholerae*, but not agglutinated by O subgroup I antiserum were considered to be of no medical importance and were called nonagglutinating or NAG vibrios. They are also known as noncholera vibrios or NCV. They have been, from time to time, associated with outbreaks of diarrhoeal or choleraic disease. Some strains of NAG vibrios produce an enterotoxin essentially similar to the cholera toxin.

Group A vibrios have been classified into over 100 subgroups based on O antigens. Subgroup I contains cholera vibrios while the others are NAG vibrios. All the subgroups possess the same H antigen.

The classification of cholera, el Tor and NAG vibrios is controversial. Taxonomists contend that all of them should belong to a single species, *V. cholerae*, subdivided into four biotypes.

V. cholerae biotype *cholerae*	— the classical cholera vibrio
V. cholerae biotype *el Tor*	— the el Tor vibrio
V. cholerae biotype *proteus*	— NAG vibrios.
V. cholerae biotype *albensis*	— water vibrios

On the other hand, it has been suggested that *V. cholerae* and *V. el Tor* should be considered as as two distinct species. For practical purposes, it is convenient to classify these vibrios under three headings — classical cholera, el Tor and NAG vibrios, the first two being biotypes, each of which consists of three serotypes — Ogawa, Inaba and Hikojima.

Bacteriophage typing schemes have been developed for cholera and el Tor vibrios. Classical cholera vibrios are classified into five types, using four phages. They are lysed without exception by Mukerjee's phage IV.

TABLE 33.6

Phage typing scheme for classical cholera vibrios

Phage type	Sensitivity to phage group			
	I	II	III	IV
1	+	+	+	+
2	–	+	+	+
3	+	–	+	+
4	–	–	+	+
5	+	+	–	+

The el Tor vibrios have been classified into six types using five phages. All strains are susceptible to phage V (Table 33.7).

A preliminary phage typing scheme has been developed for NAG vibrios. The International Reference Centre for vibrio phage typing is located at the National Institute of Cholera and Enteric Diseases, Calcutta. Many strains of vibrios produce bacteriocins (vibriocins). Vibriocin typing methods are being developed.

TABLE 33.7

Phage typing scheme for el Tor vibrios

Phage type	Sensitivity to phage group				
	I	II	III	IV	V
1	+	+	+	+	+
2	+	+	+	−	+
3	+	+	−	+	+
4	+	+	−	−	+
5	+	−	−	−	+
6	−	+	−	−	+

CHOLERA

Cholera is an acute diarrhoeal disease caused by *V. cholerae*. In its most severe form, cholera is a dramatic and terrifying illness in which profuse watery diarrhoea and vomiting may lead to hypovolaemic shock and death in less than 24 hours. In treated cases, the disease may last 4–6 days, during which period the patient may pass a total volume of liquid stool equal to twice his body weight. All of the clinical features of severe cholera result from the loss of large volumes of diarrhoeal fluid, which is bicarbonate-rich isotonic electrolyte solution. This leads to diminution of extracellular fluid volume, haemoconcentration, hypokalaemia, base-deficit acidosis and shock. The common complications are muscular cramps, renal failure, pulmonary oedema, cardiac arrhythmias and paralytic ileus.

The clinical severity of cholera varies widely, from the rapidly fatal disease to a transient asymptomatic colonisation of the intestine by the vibrios. The incidence of mild and asymptomatic infections is more with el Tor vibrios than with the classical cholera vibrios. NAG vibrios may sometimes produce a disease clinically indistinguishable from cholera. The same clinical picture may sometimes be caused by enterotoxigenic *E. coli* and, in some outbreaks, no pathogen can be identified.

The incubation period varies from less than 24 hours to about five days. The clinical illness may begin slowly with mild diarrhoea, in 1–3 days or abruptly with sudden massive diarrhoea. Although the initial diarrhoeal stool may contain faecal material, subsequent stools rapidly assume the 'rice water' appearance, with flecks of mucus in a colourless fluid. The stool has a fishy odour. Purging is painless and effortless. Vomiting, which starts along with diarrhoea, is copious, consisting of a clear, waterly fluid secreted by the upper part of the of the small intestine.

Pathogenesis: Cholera occurs only in man. Parenteral inoculation of vibrios into mice or other laboratory animals may produce a fatal bacteraemia. This bears no resemblance to the natural infection. In recent years, several animal models of cholera which resembles the human infection have been developed. In the rabbit intestinal loop model of De and Chatterjee (1953), cholera culture or a toxic filtrate is injected into the lumen of a ligated ileal loop. Within 24 hours, the loop becomes ballooned due to accumulation of fluid that resembles cholera stool in composition. The intestinal loops of many other species of animals and of chicken have been found to respond in the same manner.

Dutta and Habbu (1955) found that when infant rabbits (less than 16 days old) are infected with vibrios perorally or intraintestinally, they develop a fatal diarrhoea in 12–24 hours. The canine model, which comes closest to the human disease, was developed by Sack and Carpenter (1966). When dogs are infected with cholera vibrios through a stomach tube, after neutralisation of gastric acidity with bicarbonate, they develop vomiting and diarrhoea after an incubation period of 6–18 hours. The disease is fatal in about 90 per cent of untreated dogs.

In human infection, the vibrios enter orally through contaminated food or drink. If they survive the barrier of gastric acidity, they multiply in the alkaline contents of the small intestine. Vibrios do not penetrate deep into the gut wall and do not invade the bloodstream, though they may infect the gall bladder. The actual mechanism of the pathogenesis was clarified only in recent years.

Based on autopsy findings, it was suggested by Virchow (1897) that the vibrios caused denudation of the intestinal epithelium, and that the outpouring of fluid took place through the damaged mucosa. By 1960, it was established that the mucosa remained intact in cholera and that the changes described earlier were postmortem phenomena. It was also suggested that the fluid accumulation may be due to an exotoxin produced by the vibrio. It has now been established that cholera vibrios produce an enterotoxin which acts on the intestinal epithelium.

Cholera toxin: Cholera vibrios adhere to the intestinal epithelium, multiply and produce an exotoxin (enterotoxin, choleragen) which is responsible for the pathogenesis of cholera. It acts by the cyclic adenosine 3'5' monophosphate (cAMP) pathway. The interaction of the enterotoxin with the epithelial cells of the small intestine results in stimulation of cell bound adenyl cyclase. This leads to increased levels of cAMP in the gut epithelial cells (enterocytes), resulting in the active secretion of large volumes of fluid into the intestinal lumen. The fluid secreted is isotonic with plasma, but contains relatively more Na and HCO_3 and less Cl. Aspirin, indomethacin, chlorpromazine and ethacrynic acid inhibit the secretory effect of cholera toxin in animal models.

Besides enterotoxic activity, the cholera toxin also has the property of increasing capillary permeability. When the toxin is injected intradermally in rabbits or guinea pigs and a suitable dye, such as pontamine sky blue injected intravenously afterwards, the skin over the site of toxin injection becomes blue. This toxic activity is known as the vascular permeability factor (PF).

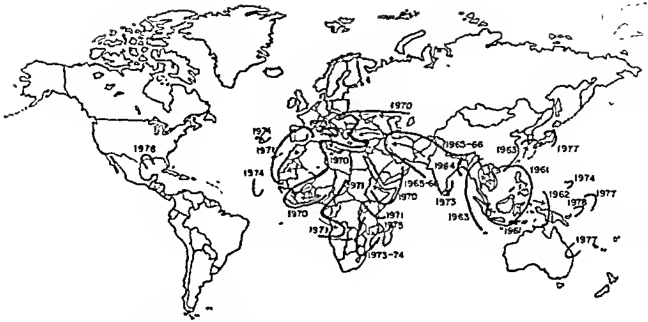
The cholera exotoxin is a protein with a molecular weight of 84,000, destroyed at an acid pH, by heating at 56 °C for 30 minutes and by treatment with pronase, but not by trypsin. It is composed of two fractions, A *(active)* and B *(binding)*. Neither fraction alone exhibits any significant toxicity, but a mixture of the two is toxic. Fraction B is the part responsible for binding to the ganglioside receptor on the host cell membrane. Fraction A can be dissociated into two polypeptide chains termed A1 and A2. The A1 subunit is the part responsible for activation of adenyl cyclase.

The exotoxin can be toxoided. Both toxin and toxoid are antigenic. The antitoxin specifically neutralises both enterotoxin and PF activities. Antitoxin is formed following natural infection with cholera vibrios, but not after immunisation with the standard bacterial vaccine. The toxin produced by the different serotypes and biotypes of cholera vibrios appears to be identical antigenically and in other respects.

Cholera culture filtrates or lysates also possess endotoxic activity. The cholera endotoxin is a lipopolysaccharide protein complex similar to the endotoxins of Gram negative intestinal bacilli. It determines the O antigenic specificity, but does not play any part in the pathogenesis of the natural disease. It is responsible for the fatal disease produced experimentally by parenteral inoculation in mice.

Epidemiology: Cholera has been endemic in the Ganges and Brahmaputra deltas in Bengal from very early times. Till the nineteenth century, cholera was confined to its endemic areas. Between 1817 and 1923 cholera spread far and wide in six pandemics, over most of the world. It was largely due to the threat of cholera pandemics that international health organisations came into being. Between 1923 and 1961, the disease remained confined to endemic areas, except for one isolated epidemic in Egypt in 1947. The most recent pandemic, the seventh, originated from a focus in Indonesia and by 1966 had encompassed the whole of Asia (Fig. 33.2).

The seventh pandemic has been caused by the el Tor vibrio in contrast to all the previous pandemics which were due to the classical cholera vibrio. There are several distinctive differences between the epidemiology of el Tor and classical cholera. Infection with el Tor vibrio leads to a larger proportion of mild cases, higher incidence of carriers and greater chances for endemicity as



SOURCE: FROM WORLD HEALTH ORGANIZATION, WEEKLY EPIDEMIOLOGICAL RECORD, SECOND SERIES

Fig. 33.2 Extension of el Tor Cholera, 1961-1978 (Courtesy Dr Paul A. Blake, Deputy Chief, Enteric Diseases Branch, CDC, Atlanta, Georgia, USA)

compared to classical cholera. The el Tor vibrio is hardier and is able to survive in the environment longer. A peculiar consequence of the seventh pandemic has been that wherever the el Tor vibrio has caused longstanding infection, in all those *areas it has displaced the classical vibrios completely*. Thus, in India, classical cholera strains are hardly ever encountered now, almost all the isolates being the el Tor type. However, in Bangladesh, classical *V. cholerae* staged a comeback in 1982 and replaced el Tor vibrios in many areas.

Epidemic cholera has a seasonal distribution, the epidemic seasons being different in different areas, but constant in any one area. Thus in Dacca, Bangladesh, the cholera season follows the monsoon and reaches a peak during November to January, but in Calcutta, the epidemic season is in the hot, dry months of March to May and the disease wanes with the onset of rains in June. In some areas of the Philippines, the cholera and monsoon peaks coincide. The reason for the seasonal patterns is not known. Epidemic cholera has been associated with fairs, festivals and other large congregations, during which sanitary *arrangements tend to be unsatisfactory*.

Two types of epidemic situations are recognised in cholera, explosive and protracted. In the explosive type, a common source or a common vehicle can usually be identified. Typically, a very large number of cases appear in a community in a short period of time. A typical explosive epidemic was the Broad Street pump episode of 1854, which was due to the pollution of water in one well and which was controlled by the London anaesthetist John Snow by the simple expedient of removing the handle from the pump. Snow's work is a classic in practical epidemiology. The protracted epidemic, on the other hand, lasts for long periods, with only a few cases per day. The

mode of transmission is not well understood, but may be due to contamination of a large body of water, such as a river.

In cholera, transmission by person to person contact is unimportant. Transmission is maintained by a cycle involving the vibrio excretor and the environment, particularly a water source. Vibrios do not survive for any considerable period in water, so the water source should be repeatedly contaminated for it to act as a prolonged source of infection. The only natural reservoir of infection is man. Both convalescent and chronic carriers exist. In the chronic carrier, the vibrio survives in the gall bladder and is shed intermittently, particularly when the carrier suffers from diarrhoea. Occasional chronic carriers may continue to shed the vibrios intermittently for several years. The half life of vibrio excretion is about four days in healthy and seven days in convalescent carriers. Fomites and vectors (flies) do not seem to be important in transmission. Relatively large doses are necessary for infection to be established, the ID50 in human volunteers being 10^6 for vibrios, as compared to 10^2 for shigella and 10^4 for *S. typhimurium.*

Cholera is essentially a disease of poverty and insanitation and affects preferentially the poorer nations and persons. The advanced nations need have little fear of cholera now because methods of effective treatment exist, and the stray imported case is unlikely to lead to an epidemic in a community with effective sanitation. During the westward spread of the seventh pandemic, the el Tor vibrio had been seeded into many European countries and in the U.S.A. and Australia, but epidemics have resulted only in the underprivileged areas. Outbreaks of el Tor cholera have occurred in Louisiana and Florida, USA, in 1978 and 1976, the source having been reported as crustaceans — crabs and shrimps. In endemic areas, cholera is seen mainly in children as adults are immune due to prior contact, while in places where cholera is newly imported, it characteristically affects adults.

Laboratory diagnosis: The importance of laboratory diagnosis of cholera is not so much for the treatment of individual cases as in facilitating cholera control by the early detection of infection and determination of the epidemiological features of an outbreak. For this to be effective, laboratory facilities should be made available to all parts of the country. This can easily be achieved by organising cholera diagnosis at various levels, ranging from the paramedical personnel for collection of appropriate specimens in the field, to the Central Reference Laboratory.

Stool, collected in the acute stage of the disease, before administration of antibiotics, is the most valuable specimen for laboratory diagnosis. Isolation of cholera vibrios from stools is a simple matter as they are present in very large numbers, 10^6—10^9 vibrios per ml. The specimen is best collected by introducing into the rectum a No. 26 or 28 rubber catheter lubricated with paraffin and letting the liquid stool flow directly into a screw-capped container. Rectal swabs may be used, provided they are made with good quality cotton wool, absorbing about 0.1–0.2 ml of fluid. They are useful in collecting specimens from convalescents who no longer have watery diarrhoea, and in such cases, the swabs should be moistened with the transport medium before sampling. Collection of stools from pans is not recommended. Vomitus is not useful. Pieces of clothing soiled with faeces and the contents of the small intestines of dead bodies may be used for culture if the situation demands.

As cholera vibrios may die in a few hours at tropical temperatures, it is necessary to preserve the specimen at 4°C or in some appropriate holding medium. Stool samples may be preserved in VR fluid or Cary-Blair medium for long periods. If the specimen can reach the laboratory in a few hours, it may be transported in enrichment media such as alkaline peptone water or Monsur's medium, thus saving the time required for isolation. If transport media are not available, strips of blotting paper may be soaked in the watery stool and sent to the laboratory packed in plastic envelopes. Whenever possible, specimens should

be plated at the bedside and the inoculated plates sent to the laboratory.

Diagnosis by direct microscopic examination of cholera stool is not recommended as the results are not reliable. For rapid diagnosis, the characteristic motility of the vibrio and its inhibition by antiserum can be demonstrated under the dark field of phase contrast microscope, using cholera stool from acute cases, or more reliably after enrichment for six hours. Demonstration of vibrios in stools by direct immunofluorescence has been attempted, but nonspecific fluorescence is common and the technique is too complicated for use in the field.

On arrival in the laboratory, the specimens sent in enrichment media should be incubated for 6–8 hours including transit time. The specimens sent in holding media should be inoculated into enrichment media, to be incubated for 6–8 hours before being streaked on a selective and a nonselective medium. It is also desirable to do direct plating before enrichment. The plating media used vary in different laboratories, but the media employed usually are bile salt agar, meat extract agar or gelatin agar for nonselective and Monsur's GTTA medium or TCBS agar for selective plates. The plates should not be older than 3–5 days and should be well dried before streaking. It is possible to identify vibrio colonies on nonselective media after incubation for 4–5 hours by examination under a stereoscope with oblique illumination. Generally, the plates are examined after overnight incubation at 37°C. Colonies suggestive of vibrios should be picked with a straight wire and tested by slide agglutination with cholera O subgroup I serum (cholera 'nondifferential' serum). If positive, agglutination should be repeated using nonspecific Ogawa and Inaba sera for serotyping. Hikojima strains will agglutinate equally well with Ogawa and Inaba sera. If agglutination is negative with one colony, it is essential to repeat the test with at least five more colonies, as agglutinable and NAG vibrios may coexist in the same specimen. If slide agglutination is positive, the isolate is tested for chick red cell agglutination. This is employed for presumptive differentiation between el Tor and classical cholera vibrios. A report can be sent at this stage, usually the day after the specimen is received. If no vibrios are isolated, a second cycle of enrichment and plating may succeed in some cases.

The isolate is then subjected to detailed study, including the oxidase test, utilisation of amino acids, lysine, arginine and ornithine, fermentation of sugars including sucrose, mannose and arabinose, haemolysis, VP, polymyxin B sensitivity and susceptibility to cholera phage IV. The strain may be sent to the International Reference Centre for vibrio phage typing at the National Institute of Cholera and Enteric Diseases (NICED) at Calcutta.

Isolates of vibrios that are not agglutinated by the O subgroup I serum should not be ignored as NAG vibrios are known to produce cholera-like disease. An antiserum to the H antigen which is shared by cholera and all NAG vibrios has been found to be a useful reagent. Any vibrio which is agglutinated by this H antiserum, but not by cholera O subgroup I antiserum, is considered to be an NAG vibrio. In the fully equipped laboratory, diagnostic tests in cholera and other diarrhoeal diseases should consist of a battery of tests designed to isolate other known pathogens also.

For isolation of vibrios from carriers, essentially the same techniques are to be followed, except that more than one cycle of enrichment may be necessary. As vibrio excretion is intermittent, repeated stool examination will yield better results. Examination of stools after a purgative (magnesium sulphate 15–30 G or Mannitol 30 G); or of bile after duodenal intubation is of special value.

Serological examination is of little use in diagnosis of cases though it may be helpful in assessing the incidence of cholera in an area. The tests available are agglutination using live or killed vibrio suspensions, indirect haemagglutination, vibriocidal test and antitoxin assay. Of these, the complement dependent vibriocidal antibody test is the most useful.

For examination of water samples for vibrios, enrichment or filtration methods may be employ-

ed. In the former, 900 ml of water are added to 100 ml tenfold concentrated peptone water at pH 9.2, incubated at 37°C for 6–8 hours and a second enrichment done before plating on selective media. For the filtration technique the water to be tested should be filtered through the Millipore membrane filter, which is then placed directly on the surface of a selective medium and incubated. Colonies appear after overnight incubation. Sewage should be diluted in saline, filtered through gauze and treated as for water.

Immunity: In cholera, the vibrios remain confined to the intestine, where they multiply and elaborate the enterotoxin, which is responsible for the disease. Immunity, therefore, may be directed against the bacterium or against the toxin — antibacterial or antitoxic. Natural infection confers some amount of immunity, but it does not seem to last for more than 6–12 months and reinfections are known after this period.

Immunisation with killed vaccines induces only antibacterial immunity. The protective effect of these vaccines, though shortlived, and especially of purified somatic antigens used as vaccines, proves that antibacterial immunity can protect against infection. The protection appears to be serotype specific, but not biotype specific. Antibacterial immunity does not appear to reduce the severity of the disease once the immunity is overcome and the disease sets in. It also does not seem to prevent the carrier state developing. Antibacterial immunity can be measured by agglutination, passive haemagglutination or vibriocidal antibody tests. These antibodies are often seen widely distributed even in areas where cholera is not endemic. These low titre antibodies may develop as a result of contact with cross reacting antigens.

Injection of the toxin or formol toxoid in experimental animals and human volunteers induces formation of antitoxin. Antitoxin may be assayed *in vivo* by neutralisation tests in intestinal loops or on rabbit skin or *in vitro* by passive haemagglutination. Antitoxic immunity has been shown to protect dogs against experimental cholera. As the toxin produced by different serotypes and biotypes of cholera vibrios is antigenically identical, immunisation with any cholera toxin would apparently protect against infection by any type of cholera vibrio. The various aspects of antitoxic immunity are under study.

Immunity may be local, in the intestine, or systemic. The appearance of local antibodies in the intestine has been known for a long time. These are known as 'copro antibodies' as they appear in the faeces. They consist of IgG, IgM and IgA.

Prophylaxis: The prevention of cholera requires essentially general measures such as provision of protected water supply and improvement of environmental sanitation. But as these are not easily attainable, vaccination continues to be the most widely used method of prevention in endemic areas.

Cholera vaccines were introduced by Ferran within a year of the discovery of the vibrio. The original vaccines were live suspensions of vibrios. As they gave rise to adverse reactions, they were replaced by killed vaccines. The vaccines routinely used now are killed suspensions containing 8000 millions *V. cholerae* per ml, composed of equal numbers of Ogawa and Inaba serotypes. Many laboratories employ classical cholera and el Tor vibrios in equal numbers in the vaccine. The concentration of the vaccine has been increased to 12,000 million per ml, in order to improve the antigenic stimulus.

Several controlled field trials have been conducted with various types of vaccines in Calcutta, Bangladesh and the Philippines. Their results indicate that the degree of protection afforded by the vaccines in current use does not exceed 50–60 per cent; the duration of protection is only 3–6 months; the rate of protection in endemic areas increases with age; a single dose of vaccine is ineffective in children below five years of age while two doses at 1–4 week intervals are highly protective; a single dose confers good protection in adults due to its acting as a booster on top of prior natural immunisation; cell-free somatic antigen preparations are as effective as whole cell vac-

cine; there is good cross protection between classical and el Tor vibrios; the cross protection between Ogawa and Inaba serotypes is doubtful and requires further study, pending which vaccine containing the homologous serotype is to be employed. Recent develnpments in killed vaccines have been the use of monovalent vaccines and of adjuvants. It has been reported that better protection may be achieved if monovalent vaccine of the serotype causing epidemics in the area is employed instead of the bivalent vaccine, but this needs confirmation. Moreover, the serotypes responsible may change unexpectedly. Oil adjuvant vaccines have been found to give more lasting protection, but they cause unacceptable local reactions. Aluminium hydroxide and phosphate adjuvant vaccines have been shown to induce a high degree of immunity, particularly in young children.

The use of live vaccines, orally, has been recommended on the ground that they give rise to antibacterial and antitoxic immunity as well as local immunity in the intestine. While attractive in concept, it has not been possible to develop suitable vaccine strains that are at the same time avirulent and capable of multiplying in the intestine after oral feeding. The development recently of a stable mutant of the cholera vibrio which produces only fraction B of the toxin without fraction A has been claimed to be a breakthrough in this respect. This strain is totally avirulent but is expected to provide local antibacterial as well as antitoxic immunity. This strain is undergoing detailed study as a potential live oral vaccine.

Trials with the cholera toxoid as a vaccine have been unsuccessful. A mixture of bacterial vaccine and toxoid is being tested for enhanced immunogenicity.

Treatment: The treatment of cholera consists essentially of the prompt and adequate replacement of lost fluid and electrolytes. The oral administration of fluid containing glucose and electrolytes, either alone or supplemented by intravenous fluids is a highly successful and freely available method of treating chnlera. Antibacterial therapy is of secondary importance. Oral tetracycline is useful in reducing the period of vibrio excretion and the need for parenteral fluids. Cholera vibrios do not appear to accept and retain R factors readily. Multiple drug resistance is not, therefore, a problem in them though drug resistant isolates have been reported from Tanzania and Bangladesh in 1979.

HALOPHILIC VIBRIOS

Vibrios that have a high requirement of sodium chloride are known as halophilic vibrios. Their natural habitat is sea water and marine life. Some

TABLE 33 8

Some characteristics of *V parahaemolyticus* and *V. alginolyticus*

	V. parahaemolyticus	*V. alginolyticus*
Indole	+	+
V.P.	−	+
Nitrate reduction	+	+
Urease	−	−
Sucrose fermentation	−	+
Swarming	−	+
Growth in 0% NaCl	−	−
7% NaCl	+	+
10% NaCl	−	+

halophilic vibrios have been shown to cause human disease — *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus*.

Vibrio parahaemolyticus

V. parahaemolyticus is the name proposed by Sakazaki (1963) for an enteropathogenic halophilic vibrio originally isolated in 1951 in Japan as the causative agent of an outbreak of food poisoning due to sea fish. Gastroenteritis due to this vibrio has since been identified in several countries and it is now considered an important cause of food poisoning throughout the world. It inhabits the coastal seas, where it is found in fishes, arthropods such as shrimps and crabs, and molluscs such as oysters. In Calcutta, it has also been found in smal pond fishes.

In morphology, it resembles the cholera vibrio, except that it is capsulated, shows bipolar staining and has a tendency to pleomorphism, especially when grown on 3% salt agar and in old cultures. Unlike other vibrios, it produces peritrichous flagella when grown on solid media. Polar flagella are formed in liquid cultures.

It grows only in media containing NaCl. It can tolerate salt concentration upto eight per cent, but not 10 per cent. The optimum salt concentration is 2–4 per cent. On TCBS agar, the colonies are green with an opaque, raised centre and flat translucent periphery The string test is positive

It is oxidase, catalase, nitrate, indole and citrate positive. Glucose, maltose, mannitol, mannose and arabinose are fermented producing acid only. Lactose, sucrose, salicin, xylose, adonitol, inositol, sorbitol are not fermented.

It is killed at 60°C in 15 minutes It does not grow at 4°C, but can survive refrigeration and freezing. Drying destroys it. It dies in distilled water or vinegar in a few minutes.

Three antigenic components have been recognised — somatic O, capsular K and flagellar H antigens. Serotyping is based on O and K antigens, 12 groups have been recognised and 39 distinct K antigens.

Not all strains of *V. parahaemolyticus* are pathogenic for man. It has been found that strains isolated from environmental sources (such as water, fish, crabs or oysters) are nearly always nonhaemolytic when grown on a special high salt blood agar (Wagatsuma agar), while strains from human patients are almost always haemolytic. This is called the Kanagawa phenomenon and is due to a heat stable haemolysin. The significance of this haemolysis is not known, but it is used as a laboratory test for pathogenicity, Kanagawa positive strains being considered pathogenic for man and negative strains nonpathogenic. No enterotoxin has been identified. The vibrio is believed to cause enteritis by invasion of the intestinal epithelium.

V. parahaemolyticus causes food poisoning associated with marine food. It also causes acute diarrhoea, unassociated with food poisoning. Abdominal pain, diarrhoea, vomiting and fever are the usual signs. Faeces contains cellular exudate and, often, also blood. Dehydration is of moderate degree and recovery occurs in one to three days. Cases are commoner in summer, and in adults than in children. In Calcutta, *V. parahaemolyticus* could be isolated from 5–10 per cent of diarrhoea cases admitted to the Infectious Diseases Hospital. *V. parahaemolyticus* is common in sea fish in some other parts of India (Andamans, Bombay, Calicut), but human cases are much less frequent.

Vibrio alginolyticus

This halophilic vibrio resembles *V. parahaemolyticus* in many respects and was formerly considered a biotype of the latter. It has a higher salt tolerance, is VP positive and ferments sucrose (Table 33.8). It is frequently found is sea fish. Its status as a human pathogen is uncertain. It has been associated with marine wound infections.

Vibrio vulnificus

V. vulnificus, previously known as L + vibrios or *Beneckea vulnifica*, is a marine vibrio of medical importance. It is VP negative and ferments

lactose but not sucrose. It has a salt tolerance of less than eight per cent. It causes two types of illness. The first is wound infection following contact of open wounds with seawater. The second type occurs in compromised hosts particularly those with liver disease. Following ingestion of the vibrio, usually in oysters, it penetrates the gut mucosa without causing gastrointestinal manifestations and enters the bloodstream, rapidly leading to septicaemia with high mortality.

AEROMONAS AND PLESIOMONAS

Besides the genus *Vibrio*, the family Vibrionaceae also contains the genera *Aeromonas* and *Plesiomonas*, some members of which have been associated with human lesions.

Aeromonas hydrophila, originally isolated from frogs, in which it causes the 'red leg disease', has been reported from many cases of diarrhoea and from some pyogenic lesions in man. *Plesiomonas shigelloides* also has been reported from diarrhoeal disease. Both these are oxidase positive, polar flagellated, Gram negative rods and may be mistaken for NAG vibrios. They may be differentiated from vibrios by biochemical tests such as utilisation of aminoacids.

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34 Pseudomonas [12].

NMB%F
12.5

The genus *Pseudomonas* consists of aerobic, nonsporing, Gram negative bacilli, motile by polar flagella. Members of this genus are mostly saprophytic, being found in water, soil or wherever decomposing organic matter is found. Some of them are pathogenic to plants, insects and reptiles. Till recently, the genus contained only one human pathogen, *Pseudomonas aeruginosa*, but lately the bacilli of glanders and melioidosis have been assigned to this genus, after prolonged taxonomical uncertainty.

Pseudomonas aeruginosa
(Ps. pyocyanea, Bacillus pyocyaneus)

Morphology. It is a slender Gram negative bacillus, 1.5–3 $\mu \times 0.5\mu$, actively motile by a polar flagellum. Occasional strains have two or three flagella. It is noncapsulated, though mucoid strains may sometimes occur. When grown in the absence of sucrose, an extracellular polysaccharide slime layer may be formed: Strains isolated from clinical specimens frequently possess pili

Cultural characteristics: It is an obligate aerobe. Growth occurs at a wide range of temperatures, 5°C–42°C, the optimum being 37°C. It grows well on ordinary media, producing large, opaque, irregular colonies, with a distinctive musty, mawkish or earthy smell. Iridescent patches with a metallic sheen are seen in cultures on nutrient agar. Crystals are seen beneath the patches. It grows on MacConkey and DCA media, forming nonlactose fermenting colonies. Many strains are haemolytic on blood agar. In broth, it forms a dense turbidity with a surface pellicle.

Ps. aeruginosa produces a number of pigments, the best known being pyocyanin and fluorescin. Pyocyanin is a bluish green phenazine pigment soluble in water and chloroform. Fluorescin is a greenish yellow pigment soluble in water but not in chloroform. In old cultures it may be oxidised to a yellowish brown pigment. Pyocyanin is produced only by *Ps. aeruginosa*, but fluorescin may be produced by many other species also. Other pigments produced are pyoverdin, pyorubin and pyomelanin in various combinations Some strains may be nonpigmented. It is not known whether the pigments have any role in pathogenesis. Some of the pigments, particularly pyocyanin, inhibit the growth of many other bacteria and may therefore contribute to *Ps. aeruginosa* emerging as the dominant bacterium in mixed infections.

Biochemical reactions: Metabolism is oxidative and not fermentative. Peptone water sugars are unsuitable for detecting acid production, since this is weak and gets neutralised by alkali produced from peptone. An ammonium salts medium in which the sugar is the only carbon source is the best. Glucose is utilised oxidatively, forming acid only. Indole, MR, VP and H_2S tests are negative. Nitrates are reduced to nitrites and further to gaseous nitrogen. Catalase, oxidase and arginine dihydrolase are positive. IMV

Classification As *Ps. aeruginosa* has become a very important cause of hospital infections, its

classification is essential for epidemiological purposes. Serotyping, pyocin (bacteriocin) typing and bacteriophage typing have been used but no method of classification bas been entirely satisfactory. Based on the O antigen, it has been divided into 27 serotypes using slide agglutination. Pyocin typing is the method most frequently used for the classification of isolates. Bacteriophage typing schemes have been proposed but have not been widely accepted.

Resistance: The bacillus is not particularly heat resistant, being killed at 55°C in one hour, but exhibits a high degree of resistance to chemical agents. It is resistant to the common antiseptics and disinfectants such as quaternary ammonium compounds, chloroxylenol and hexachlorophane, and may even grow profusely in bottles of such antiseptic lotions kept for use in hospitals. Indeed, selective media have been devised for *Ps. aeruginosa* incorporating dettol or cetrimide.

Ps. aeruginosa possesses a considerable degree of natural resistance to antibiotics. Polymyxin B, colistin, gentamycin, amikacin, carbenicellin, piperacellin and cefoxamine are effective against most strains. Some strains may be sensitive *in vitro* to streptomycin and tetracycline, but they are generally of little therapeutic use.

Pathogenicity: 'Blue pus' was known as a surgical entity long before Gessard (1882) isolated *Ps. aeruginosa* from such cases. Both the specific names of the bacillus refer to its capacity to cause 'blue pus', the term *aeruginosa*, meaning verdigris which is bluish green in colour and *pyocyanea*, being a literal translation of 'blue pus'.

The pathogenic importance of the bacillus was not adequately recognised till recently, when it has established itself as one of the most troublesome agents causing nosocomial infections. In the community outside the hospital, the commonest infection caused by *Ps. aeruginosa*, is suppurative otitis, which is chronic though not disabling. In the hospital, it may cause localised or generalised infections. Localised lesions are commonly infections of wounds and bedsores, eye infections and urinary tract infections following catheterisation. *Ps. aeruginosa* is the commonest and most serious cause of infection in burns. It is also one of the commonest agents responsible for iatrogenic meningitis following lumbar puncture. It frequently causes post-tracheostomy pulmonary infection. Septicaemia occurs in patients who are debilitated due to concomitant infection, malignancy or immunosuppressive therapy. Ecthyma gangrenosum and many other types of skin lesions have been described, occurring either alone or as part of generalised infection, mainly in patients with leukaemia and other types of malignancy.

Ps. aeruginosa had been described as one of the agents responsible for infantile diarrhoea. Evidence has now been presented that strains isolated from outbreaks of diarrhoea may form a heat labile enterotoxin and give a positive rabbit ileal loop reaction.

The preeminent role of *Ps. aeruginosa* in hospital infection is due to its resistance to common antibiotics and antiseptics, and its ability to establish itself widely in hospitals. Equipment such as respirators, articles such as bed pans and medicines such as lotions, ointments and eye drops may be frequently contaminated. *Ps. aeruginosa* is carried in the intestines of a small proportion of healthy persons in the community, but the incidence rises steeply following hospitalisation.

The mechanisms of pathogenesis are not clearly understood. It has been claimed that the pathological processes seen in infection are caused by the extracellular products of the bacterium. Several extracellular products have been identified in the culture filtrates. Exotoxin A is a lethal toxin which functions as NADase, acting like the diphtheria toxin. Several proteases are produced by the bacillus. Elastases may be responsible for haemorrhagic lesions in skin infections and the destruction of corneal tissue in eye infections. Two haemolysins are produced, one a phospholipase and the other a glycolipid. The former acts on the lung tissue causing atelectasis and necrosis, facilitating invasion of the lung in pneumonia. The enterotoxin causes diarrhoeal

Y. pestis
Yer. Y. pseudotuber.
Y. enterocolitica
Pasteurella ← Past. p. multocida
Fran. p. aviseptica.
F. tularensis.

35 Pasteurella, Yersinia, Francisella

The plague bacillus and many other Gram negative, short bacilli that are primary pathogens of rodents were grouped together in the genus Pasteurella. Based on cultural and biochemical differences, this group has been divided into three genera — Yersinia, Pasteurella and Francisella. The genus Yersinia, containing the medically important species *Y. pestis* (the causative agent of plague), *Y. pseudotuberculosis* (a primary pathogen of rodents) and *Y. enterocolitica* (which causes enteric and systemic disease in animals and man), was so named after Alexandre Yersin, who discovered the plague bacillus. The genus *Yersinia* is now assigned to the family Enterobacteriaceae. The genus Pasteurella contains several related bacteria causing haemorrhagic septicaemia in different species of animals and occasionally producing local and systemic infections in man, grouped under a common species named *P. multocida*. One of these, *P. aviseptica* is the chicken cholera bacillus used by Pasteur for the development of the first attenuated bacterial vaccine. Hence the name Pasteurella. The genus Francisella, consisting of the single species, *F. tularensis* is named after Francis for his pioneering studies on tularaemia, caused by this bacillus

Yersinia pestis
(Pasteurella pestis)

The plague bacillus was discovered independently and simultaneously by Yersin and Kitosato (1894) in Hong Kong at the beginning of the last pandemic of the disease.

Morphology: *Y. pestis* is a short, plump, ovoid, Gram negative bacillus, about 1.5 μ x 0.7 μ in size, with rounded ends and convex sides, arranged singly, in short chains or in small groups. In smears stained with methylene blue, it shows bipolar staining (safety pin appearance) with the two ends densely stained and the central area clear. Pleomorphism is very common and in old cultures, involution forms are seen — coccoid, club shaped, filamentous and giant forms. Pleomorphism is characteristically enhanced in media containing 3% NaCl (Fig. 35.1).

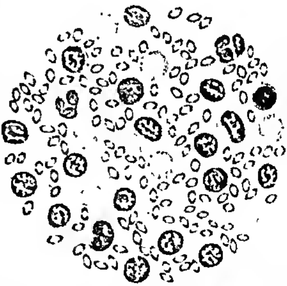

Fig. 35.1 Smear from gland puncture in a case of plague showing *Y. pestis* with bipolar staining (safety pin appearance), a few red blood cells and lymphocytes

The bacillus is surrounded by a slime layer (envelope or capsule). It is nonmotile, nonsporing and nonacid fast.

Cultural characteristics: The plague bacillus is aerobic and facultatively anaerobic. Growth occurs over a wide range of pH (pH5–9.6, optimum pH 7.2) and temperature (range 2°C–45°C). The optimum temperature for growth (unlike most pathogens) is 27°C, but the envelope develops best at 37°C.

It is not nutritionally exacting and grows on ordinary media. On nutrient agar, colonies are small, delicate, transparent discs, becoming opaque on continued incubation. Colonies on blood agar or other haemin containing media are dark brown due to the absorption of the haemin pigment. Colourless colonies are formed on MacConkey agar. In broth, a flocculent growth occurs at the bottom and along the sides of the tube, with little or no turbidity. A delicate pellicle may form later. If grown in a flask of broth with oil or ghee floated on the top (ghee broth), a characteristic growth occurs which hangs down into the broth from the surface, resembling stalactites (stalactite growth) (Fig. 35.2).

Fig 35.2 *Y. pestis* in ghee broth culture. Stalactite growth

Biochemical reactions: Glucose, maltose and mannitol, but not lactose or sucrose, are fermented with the production of acid, but no gas. Indole is not produced. It is MR positive and VP and citrate negative, catalase positive and oxidase and urease negative. Gelatin is not liquefied. Based on the fermentation of glycerol and reduction of nitrate, Devignat has distinguished three physiological varieties of *Y. pestis*. This typing appears to be of epidemiological sig-

ImViC → - + - -

TABLE 35.1

Biotypes of *Yersinia pestis*

Variety	Glycerol fermentation	Nitrate reduction	Geographical distribution
Y. pestis var. *orientalis*	–	+	Primary foci in India, Burma and China. Causative agent of 1894 pandemic. Responsible for wild plague in Western USA, South America, South Africa.
Y. pestis var. *antiqua*	+	+	Transbaikalia, Mongolia, Manchuria. Perhaps responsible for the Justinian Plague.
Y. pestis var. *mediaevalis*	+	–	South East Russia.

nificance because of the different geographical distribution of the types (Table 35.1).

Resistance: The plague bacillus is easily destroyed by exposure to heat, sunlight, drying and chemical disinfectants. It is destroyed by heat at 55°C or by 0.5% phenol in 15 minutes. It remains viable for long periods in cold, moist environments. It can survive for several months, and even multiply, in the soil of rodent burrows. All strains are lysed by a specific antiplague bacteriophage at 22°C.

Antigens, toxins and other virulence factors. Plague bacilli are antigenically homogenous and serotypes do not exist. The antigenic structure is complex. At least 20 antigens have been detected by gel diffusion and biochemical analysis. Many of them have been claimed to be virulence factors. They include the following:

1. A heat labile protein envelope antigen (Fraction I or F–I) best formed in cultures incubated at 37°C. It inhibits phagocytosis and is generally present only in virulent strains. This antigen has therefore been considered a virulence determinant, but occasional strains deficient in Fraction I antigen have been isolated from fatal human cases. The antibody to this antigen is protective in mice.

2. Two antigens, designated V and W and always produced together, have been considered to be the virulence factors as they inhibit phagocytosis.

3. Virulent strains produce a bacteriocin (Pesticin I); coagulase and fibrinolysin. Pesticin I inhibits strains of *Y. pseudotuberculosis*, *Y. enterocolitica* and *E. coli*.

The term 'plague toxins' refers to at least two classes of toxins found in culture filtrates or cell lysates. The first is the endotoxin, a lipopolysaccharide similar to the endotoxins of enteric bacilli. The second class of toxins is protein in nature, possessing some properties of both exotoxins and endotoxins. They are thermolabile and may be toxoided, but do not diffuse freely into the medium and are released only by the lysis of the cell. They are called 'murine toxins' as they are active in rats and mice, but not in guinea pigs, rabbits and primates. On injection into experimental animals, plague toxins produce local oedema and necrosis with systemic effects on the peripheral vascular system and liver. The role of plague toxins in natural disease in man is not known.

4. Virulence also appears to be associated with an unidentified surface component which absorbs haemin and basic aromatic dyes in culture media to form coloured colonies.

5. Virulence has also been associated with the ability for purine synthesis.

PLAGUE

Plague is an ancient scourge of mankind. The disease was familiar to the ancient civilisations of Asia. The Bhagavata Purana urged householders to flee when rat falls were noticed.

Central Asia is believed to have been the original home of plague, from where it has, in wave after wave, spread far and wide, causing epidemics and pandemics, exacting a toll of human life surpassing any other disease. The identity of the Biblical plague of the Philistines (1320 B.C) is in doubt, but the pandemic that occurred during the reign of Emperor Justinian (542 A.D.) was undoubtedly bubonic plague and caused a hundred million deaths. In the 14th century, pandemic plague known as the 'Black death' is believed to have killed a quarter of all mankind. Historians of plague identify 41 epidemics before the birth of Christ and 109 epidemics in the next 15 centuries. There are records of 45 pandemics between 1500 and 1720 A.D. The disease was quiescent in the 18th and 19th centuries and confined to endemic foci. The last pandemic started in Hong Kong in 1894 and spread throughout the world. India was one of the countries worst hit by this pandemic. Plague reached Bombay in 1896 and spread all over the country during the next few years, causing more than 10 million deaths by 1918. It gradually receded thereafter. During 1958–77, Plague occurred in 29 countries involving over 45,000

patients. Cases were reported practically every year from Bolivia, Brazil, Burma, Ecuador, Malagasay, Peru, USA, Vietnam and Zaire. The war in Vietnam involving major defoliation operations led to a marked incidence of plague during the late 1960s. In 1974 plague began to increase in Burma, involving virtually the whole country. India has been free of the disease since 1967. In 1981, only 191 cases and 24 deaths were reported throughout the world. Plague survives in several scattered natural foci (Fig. 35.3)

In man, plague occurs mainly in three forms, bubonic, pneumonic and septicaemic. In bubonic plague, after an incubation period of 2–5 days, the lymph nodes draining the site of entry of the bacillus become infected. As the plague bacillus usually enters through flea bites on the legs, the inguinal nodes are involved and hence the name 'bubonic' (*bubon*, meaning groin). The gland becomes enlarged and suppurates. The bacilli enter the bloodstream and produce septicaemia. Sometimes there are haemorrhages into the skin and mucosa. The case fatality in untreated cases varies from 30–90 per cent.

2) Plague pneumonia may sometimes occur during epidemics of bubonic plague. Rarely, primary pneumonic plague may occur in epidemic form, as happened in Manchuria during 1910–1912, causing some 60,000 deaths. Pneumonic plague is spread by droplet infection. The bacilli spread through the lymphatics producing haemorrhagic pneumonia. Cyanosis is very prominent. The bloody mucoid sputum that is coughed out contains bacilli in enormous numbers. Pneumonic plague is highly infectious and, in untreated patients, almost invariably fatal.

3) Septicaemic plague is usually the terminal event in bubonic or pneumonic plague, but may sometimes occur primarily. Cases of mild plague (*Pestis minor*) are seen in some epidemics. Meningitic involvement may occur rarely. Human carriers have not been recorded, but asymptomatic infection of the throat has been observed in some contacts.

Epidemiology: Plague is a zoonotic disease. The plague bacillus is naturally parasitic in rodents. Infection is transmitted among them by rat fleas,

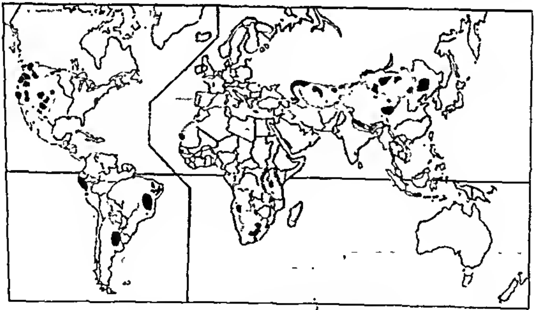

Fig. 35.3 Natural foci of plague, known and suspected
■ Known focus ▭ Probable plague area

The fleas acquire the infection by feeding on infected rodents. In the flea, the bacilli multiply in the stomach to such an extent that they block the proventriculus. The interval between the ingestion of infected blood and blocking in the proventriculus is the extrinsic incubation period, which is usually about two weeks in *Xenopsylla cheopis*. When such a 'blocked flea' bites another rodent, it cannot suck in blood because the bacterial mass blocks the passage mechanically. The blood, mixed with the bacteria is regurgitated into the bite, transmitting the infection. Infection may also be transferred by contamination of the bite wound with the faeces of infected fleas. When a diseased rat dies (rat fall), the fleas leave the carcass and in the absence of another rat, may bite man, causing bubonic plague.

MCQ Several species of fleas may act as vectors, the most important being *Xenopsylla cheopis*, *X. astia* and *Ceratophyllus fasciatus*. *X. cheopis*, the predominant species in North India, is a more efficient vector than the South Indian species, *X. astia*. This has contributed to the more extensive nature of plague outbreaks in the North as compared to South India. Plague epidemics generally occur in the cool, humid seasons that favour the multiplication of fleas, leading to a high 'flea index' (mean number of fleas per rat). In the hot, dry weather, fleas do not thrive and the transmission of infection is interrupted.

The studies of the various Governmental Plague Commissions in Bombay, during the early years of this century, helped to clarify the epidemiology of plague. It was found that plague produced epizootics first in *Rattus norvegicus* (sewer rat). When their numbers dwindled, the disease passed to the domestic rat, *R. rattus*. It was from the domestic rat that the infection spread to man.

Two natural cycles of plague exist, the domestic and the wild plague. The term domestic plague refers to plague that is intimately associated with man and rodents living with man, possessing a definite potential for producing epidemics. 'Wild plague' (formerly also called sylvatic plague) occurs in nature in wild rodents, independent of man. The rodents involved vary in different regions. Over 200 species and subspecies are involved. In Western U.S.A., prairie dogs, ground squirrels, wood rats and mice are found infected. In the endemic areas of the U.S.A., cases of human plague have occurred following contact with wild animals, and even with domestic carnivores, particularly pet cats. In Java, the field rat is the reservoir. In India, the gerbil (*Tatera indica*) and the handicoot are infected.

Attenuated *Y. pestis* organisms have been isolated from natural foci. These organisms may produce infection without causing death. It is not known whether these organisms reacquire virulence.

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Laboratory diagnosis: The laboratory should be able to diagnose plague not only in man, but in rodents also, as timely detection of infection in rats may help to prevent epidemic spread.

A rat which died of plague may carry infected fleas and should be handled with care. Pouring kerosene oil over the carcass is a simple method of eliminating the fleas. In the laboratory, the carcass should be dipped in 3% lysol to destroy ectoparasites. Rats:

During epizootics, it is easy to diagnose plague in rats. Buboes are present usually in the cervical region. They are hard and can be moved under the skin. On section, the bubo may show congestion, haemorrhagic points or grey necrosis. Smears from the bubo stained with methylene blue show the bipolar stained bacilli. The fluorescent antibody technique may be of use in identifying plague bacilli in the impression films of the tissues. Bacilli in bubo show considerable pleomorphism. The liver is mottled, with red, yellow or grey stippling. The spleen is enlarged and moulded over the stomach, with granules or nodules on the surface. A characteristic feature is pleural effusion, which may be clear, abundant and straw coloured, or less often, blood stained. Bacilli may be demonstrated microscopically in spleen smears and heart blood also. Cultures may be made from the buboes, spleen, heart blood

and, particularly, from bone marrow in decomposed carcasses.

In badly putrified carcasses, microscopy and culture may not be successful. The putrified tissue may be rubbed on the shaven abdomen of a guinea pig. The plague bacillus is able to penetrate through the minute abrasions caused by shaving and initiate lethal infection. Diagnosis, in such cases, may also be established by the thermo-precipitation test. The tissue, mixed with 5–10 parts of distilled water, is boiled for five minutes, filtered and the clear filtrate layered on antiplague serum in a narrow test tube. In positive cases, a precipitate appears at the interface after five minutes incubation at 37°C, increasing to a maximum in two hours.

Diagnosis of sporadic plague in rats may be difficult. Success is achieved by a combination of culture and animal inoculation, using pooled organs. The bacillus may also be isolated from pooled fleas. The only serological test recommended is passive haemagglutination, using tanned sheep erythrocytes sensitised with Fraction 1 antigen.

In human bubonic plague, a small vesicle may be present at the site of entry of the bacillus in early cases and bacilli may be demonstrated in the vesicle fluid. Bacilli may be readily demonstrated in buboes by microscopy, culture or animal inoculation. Blood cultures are often positive.

3) In pneumonic plague, the bacilli can be demonstrated in the sputum by microscopy, culture or animal inoculation.

Serological tests are sometimes useful in diagnosis. Antibodies to F–I antigen may be detected by agglutination or complement fixation tests The latter test may be used also for detecting the antigen in tissues. Complement fixing antibodies decrease rapidly during convalescence. The passive haemagglutination test, using tanned erythrocytes coated with F–I antigen or murine toxin, is useful for identifying plague foci, as the test remains positive for several years after recovery from plague.

Prophylaxis: In the prevention of domestic plague, general measures such as control of fleas and rodents are of great importance. Specific protection may be provided by vaccines. Two types of vaccines have been in use 1) killed and 2) live attenuated vaccines. The killed vaccine used in India (prepared at the Haffkine Institute, Bombay) is a whole culture antigen. A virulent strain of the plague bacillus is grown in casein hydrolysate broth for 2–4 weeks at 32°C and killed by 0.05 per cent formaldehyde and preserved with phenyl mercuric nitrate (Sokhey's modification of Haffkine's vaccine). Standardisation is by immunogenic potency rather than by bacterial counts. The two avirulent strains used for the preparation of live vaccines are Otten's Tjiwidej strain from Java and Girard's EV strain from Malagasay. Since live vaccines are difficult to prepare and to distribute in large quantities and may provoke unacceptable reactions, killed vaccines are recommended for general use. Vaccination may reduce the morbidity and mortality to some extent in bubonic plague, but not in pneumonic plague. The immunity following vaccination does not last for more than six months. On the other hand, an attack of plague confers lasting immunity. This is due to a tissue immunity rather than a humoral one. Mass vaccination is not recommended against plague now.

In persons at special risk, chemoprophylaxis may be employed for temporary protection. Tetracycline is the drug recommended, with sulphonamides as the next alternative.

Treatment: Early treatment is essential because of the rapid progression and high fatality of the disease. Tetracycline is the recommended drug for both bubonic and pneumonic plague. Streptomycin is very effective, but severe intoxication may occur due to massive destruction of bacilli and release of toxic products. Strains resistant to streptomycin have been observed in the Far East. 4) Chloramphenicol and Kanamycin are effective. The antiplague serum, once employed in the treatment of severe cases, is no longer recommended.

Yersinia pseudotuberculosis *(Pasteurella pseudotuberculosis)*

This bacillus resembles the plague bacillus closely, but can be distinguished by its relatively poor growth on MacConkey's agar, motility at 22°C (but not at 37°C), production of urease, fermentation of rhamnose and melibiose and failure to be lysed by antiplague bacteriophage at 22°C. Distinction between *Y. pseudotuberculosis* and *Y. pestis* becomes important when the former is isolated from rats.

Y. pseudotuberculosis is antigenically heterogeneous, six serological groups and nine serotypes being distinguished, based on somatic and flagellar antigens. It shows antigenic cross relationships with *Y. pestis* as well as salmonellae.

The natural mode of infection in animals is probably by the alimentary tract. In infected guinea pigs, the liver, spleen and lungs show multiple nodules resembling tuberculosis lesions (hence the name pseudotuberculosis). Human infection occurs rarely and may present as a fatal typhoid-like illness with hepatosplenomegaly and purpura or as mesenteric lymphadenitis simulating acute appendicitis. It has also been reported to cause erythema nodosum and gastroenteritis.

Yersinia enterocolitica

This bacillus resembles *Y. pseudotuberculosis* in being motile at 22°C, but differs from it in fermenting sucrose and cellobiose and decarboxylating ornithine. It does not ferment rhamnose or melibiose. Many strains give a positive VP test and form indole. Five biotypes have been identified based on cultural and biochemical characteristics. The antigenic structure of *Y. enterocolitica* is distinct from that of *Y. pseudotuberculosis*. More that 50 O serotypes and 19 H factors have been reported. Most human isolates belong to serotypes, 03, 08 and 09. Serological cross reactions between serotypes 9 and Brucella occur.

Y. enterocolitica has been isolated from a wide range of domestic and wild animals, and in recent years, is increasingly being reported from human clinical material. It produces three types of disease in humans. The first occurs in young children as self-limited gastroenteritis which may be either inflammatory or noninflammatory. The second is mesenteric adenitis and inflammatory terminal ileitis in older children, that may mimic appendicitis. The third category is systemic disease, typically in adults, often characterised by bacteraemia, meningitis, arthralgia or erythema nodosum.

The term 'yersiniosis' denotes infection with yersiniae other than *Y. pestis*. These are zoonoses and human infection appears to be acquired accidentally from disease cycles of wild or domesticated animals.

TABLE 35.2
Some differentiating features among Yersinia and Pasteurella

	Y. pestis	*Y. pseudo-tuberculosis*	*Y. entero-colitica*	*P. multocida*
Motility at 22°C	−	+	+	−
Growth on MacConkey agar	+	+	+	−
Acid from sucrose	−	−	+	+
Acid from maltose	+	+	+	−
Indole	−	−	∓	+
Oxidase	−	−	−	+
Urease	−	+	+	−
Ornithine decarboxylase	−	−	+	+

Pasteurella multocida
(Pasteurella septica)

A group of related bacteria isolated from haemorrhagic septicaemia in a variety of animals and birds had, in the past, been named according to their species of origin — *P. boviseptica, lepiseptica, aviseptica,* etc. Though they show some degree of host specificity, they are so alike in other respects that they are now considered strains of a single species designated *P. multocida.*

P. multocida is a nonmotile, Gram negative bacillus generally resembling Yersinia, but differing in being oxidase positive, producing indole and failing to grow on MacConkey's agar.

The bacillus is often carried in the upper respiratory tract of a variety of animals such as dogs, cats, rats, cattle and sheep. It may sometimes occur as a commensal in the human respiratory tract also. Human infection is rare, but may occur following animal bites or trauma. The clinical manifestations may be local suppuration following animal bites (wound infection, cellulitis, abscess, osteomyelitis), meningitis following head injury, respiratory tract infection (pneumonia, bronchitis, sinusitis) or appendicitis and appendiceal abscess.

The bacillus is sensitive to tetracycline and streptomycin, and most strains to penicillin as well.

Francisella tularensis
(Pasteurella tularensis; Brucella tularensis)

This is the causative agent of tularaemia, a disease of rabbits and other rodents, originally described in Tulare County, California. Infection is transmitted by ticks and several other arthropod vectors. Human infection may occur by direct contact with infected rodents such as rabbits. Infection can also be acquired by ingestion of contaminated meat or water and inhalation of infective aerosols.

It is a minute, capsulated, nonmotile, Gram negative bacillus, about 0.3–0.7 $\mu \times 0.2\ \mu$ in size. It resembles mycoplasma in being filterable and in multiplying by filament formation and budding, besides binary fission. In infected animals, it acts as an intracellular parasite, being found in large masses inside the liver and spleen cells. It has fastidious growth requirements and special media such as Francis' blood dextrose cystine agar have to be employed for its isolation. Minute transparent colonies appear after incubation for 3–5 days.

In man, tularaemia may present as a local ulceration with lymphadenitis, a typhoid-like fever with glandular enlargement or an influenza-like respiratory infection. The disease may also be water borne, as a result of water pollution with excreta of infected rodents. The bacillus is highly infectious and laboratory infection has been quite common. Diagnosis may be made by culture or by inoculation into guinea pigs or mice. Agglutinating antibodies may be demonstrated in sera from patients.

The disease has been reported from America, Europe and Japan. An attenuated vaccine is available which can be administered by scarification to persons who are subject to high risk of infection.

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36 Haemophilus

The genus *Haemophilus* contains small, nonmotile, nonsporing, Gram negative bacilli that are parasitic on man or animals. They are characterised by their requirement of one or both of two accessory growth factors (X and V) present in blood (*Haemophilus*, meaning blood loving).

Pfeiffer (1892) observed that a small, Gram negative bacillus was 'constantly present' in the sputum of patients from the influenza pandemic of 1889–1892 and proposed this as the causative agent of human influenza. This came to be known as the 'influenza bacillus' (Pfeiffer's bacillus), later renamed *Haemophilus influenzae*. The causal relationship between this bacillus and human influenza could not be substantiated and was finally disproved when Smith, Andrewes and Laidlaw (1933) isolated the influenza virus.

Many other bacilli growing preferentially in media containing blood came to be included in the genus *Haemophilus*. But as some of them (e.g. whooping cough bacillus, Morax-Axenfield bacillus) are not dependent on X and V factors, they have been removed from the genus.

Haemophilus influenzae
(Influenza bacillus; Pfeiffer's bacillus)

Morphology: *H. influenzae* is a small ($1.5\ \mu \times 0.3\ \mu$) Gram negative, nonmotile, nonsporing bacillus, exhibiting considerable pleomorphism (Fig. 36.1). In sputum, it usually occurs as clusters of coccobacillary forms, while in CSF from meningitis cases, long, bacillary and filamentous forms predominate. Cells from young cultures (18–24 hours) are usually coccobacillary, while older cultures are distinctly pleomorphic. Strains isolated from acute infections are often capsulated.

The bacilli are relatively difficult to stain. Staining for 5–15 minutes with Loeffler's methylene blue or dilute carbol fuchsin gives good results.

Cultural characteristics: The bacillus has fastidious growth requirements. The accessory growth factors (bacterial vitamins) named X and V present in blood are essential for growth. The X factor is a heat stable iron-porphyrin-haematin or other haemins. It is necessary for the synthesis of catalase and other enzymes involved in aerobic respiration. Some strains do not require the X factor for anaerobic growth. The V factor is a heat labile (destroyed at 120°C in a few minutes) factor, present in red blood cells and in many other animal and plant cells. It is synthesised by some fungi and bacteria (e.g., *Staph. aureus*) in excess of their requirements and released into the surrounding medium. The V factor can be supplied as coenzyme I or II or nicotinamide adenine dinucleotide (NAD). It appears to act as a hydrogen acceptor in the metabolism of the cell.

It is aerobic, but grows anaerobically also, the optimum temperature being 37°C. Some strains require 10% CO_2. It grows on blood agar, but growth is scanty, as the V factor is not freely available, being imprisoned inside the red blood cells. Growth is, therefore, better if a source of the V factor is also provided. When *Staph. aureus* is streaked across a plate of blood agar on which a specimen containing *H. influenzae* has been

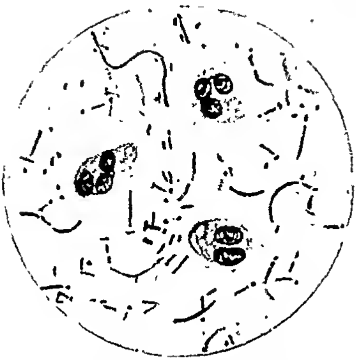

Fig 36.1 *H. influenzae* in cerebrospinal fluid showing pleomorphism

inoculated, after overnight incubation, the colonies of *H. influenzae* will be large and well developed alongside the streak of staphylococcus, and smaller farther away. This phenomenon is called 'satellitism' and demonstrates the dependence of *H. influenzae* on the V factor, which is available in high concentration near the staphylococcal growth and only in smaller quantities away from it. This is a routine test in clinical bacteriology for the identification of *H. influenzae* (Fig. 36.2).

When blood agar is heated to 80°C – 90°C until it turns chocolate in colour (chocolate agar), or boiled for a few minutes (boiled blood agar), the V factor is released from within the erythrocytes and hence these media are superior to plain blood agar for growing *H. influenzae*. Clear transparent media may be prepared by boiling and filtering a mixture of blood and nutrient broth (Levinthal's medium), or by adding a peptic digest of blood to nutrient agar (Fildes agar). Fildes agar is best for primary isolation of *H. influenzae* and it gives a copious growth. Capsulated strains produce translucent colonies with a distinctive iridescence on Levinthal's agar.

Biochemical reactions. Fermentation reactions are irregular. Nitrates are reduced to nitrites and some strains form indole. Biochemical reactions are not helpful in identification or classification

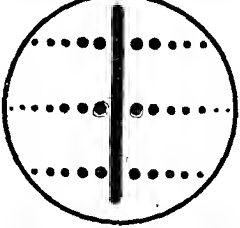

Fig 36.2 Satellitism. *H. influenzae* colonies are large near growth of staphylococcus, and smaller away from it.

Resistance: *H. influenzae* is a delicate bacterium, readily destroyed by heating (55°C for 30 minutes), refrigeration (0°C–4°C), drying and disinfectants. In cultures, the cells die within two or three days due to autolysis. Cultures may be preserved for about a month on chocolate agar slopes in screw capped bottles. For longterm preservation, the cultures may be lyophilised.

Antigenic properties: Isolates occur as one of two major colony types — the small granular (R or Rough) colony produced by the noncapsulated strains from the respiratory tract and the mucoid iridescent (S or smooth) colony of capsulated strains from the sites of Invasive lesions. Noncapsulated strains are antigenically heterogeneous. Some somatic antigens have been identified, but they are of no practical significance. Capsulated strains have been classified by Pittman into six types, designated types *a* to *f*, based on capsular antigens, which are polysaccharide in nature and resemble pneumococcal capsular antigens. Type *b* strains account for most infections. Type *b* antigens can be detected in body fluids including the urine of infected patients. It is immunochemically similar to the K antigen of *E. coli*. Typing is done by agglutination, the 'Quellung test' or by precipitation of the extracted polysaccharide with specific antisera.

Variation: Colonies show a smooth-to-roug (S→R) variation, associated with loss of capsul and virulence. As in the case of pneumococc genetic transformation has been demonstrate with *H. influenzae* also. The characters tran formed are the capsular antigen type and antibi tic resistance.

Pathogenicity: *H. influenzae* is not naturally pathogenic for animals, but intraperitoneal inoculation of large doses is fatal in mice, guinea pigs and rabbits. This is probably due to the endotoxin. Intracerebral inoculation of some strains, or intraperitoneal inoculation of the bacilli suspended in hog gastric mucin may lead to a fatal infection with blood invasion. Capsulated strains are more virulent than noncapsulated strains.

Diseases due to *H. influenzae* may be considered under two groups. In one group, the bacillus acts as a primary pathogen, causing suppurative infections. Haemophilus meningitis is the most important infection in this group, others being laryngoepiglottitis, otitis media, pneumonia, arthritis, endocarditis and pericarditis. These infections are usually seen in children and are caused by capsulated strains, type *b* accounting for most cases. In the second group, the bacillus causes secondary or superadded infections, usu-

Gp I → Primary pathogen
supp.
Gp II

TABLE 36.1

Growth characteristics of Haemophilus species

Species	Growth requirements		Haemolysis
	X Factor	V Factor	
H. influenzae	+	+	−
H. aegypticus	+	+	−
H. suis	+	+	−
H. haemolyticus	+	+	+
H. ducreyi	+	−	+
H. aphrophilus	+	−	−
H. parainfluenzae	−	+	±

ally of the respiratory tract. These include chronic bronchitis, bronchiectasis and sinusitis. These are usually seen in adults and are caused by noncapsulated strains.

Meningitis: This is the most serious disease produced by *H. influenzae* with case fatality rates of about 90 per cent in the untreated. The bacilli reach the meninges from the nasopharynx, apparently through the bloodstream and initiate a purulent meningitis. The large majority are due to type *b* strains. The disease is commoner in children, between two months and three years of age. This age incidence has been correlated with the absence of bactericidal antibodies. Older children develop immunity as a result of subclinical infection. It has been reported recently that in North America, the percentage of adults without bactericidal antibodies has been increasing. This has led to an increase in haemophilus meningitis in adults.

>2yr

Laryngoepiglottitis (Croup): This is an acute inflammation of the epiglottis with obstructive laryngitis, seen in children over two years old. Untreated cases may be fatal within hours. Tracheostomy is often necessary to relieve respiratory obstruction caused by the grossly enlarged uvula. This condition is always associated with bacteraemia and blood cultures are usually positive. >2y

Pneumonia: Haemophilus pneumonia typically occurs in infants and is accompanied by empyema and sometimes meningitis as well. In older children and adults, the picture is of lobar pneumonia. While these are primary infections due to capsulated strains, bronchopneumonia may occur as a secondary infection with noncapsulated strains. *H. influenzae* was a frequent cause of fatal pneumonia in the pandemic of influenza in 1918–1919 but this association has not been found in recent years.

Suppurative lesions: Suppurative lesions such as arthritis, endocarditis, pericarditis may result from haematogenous dissemination. Otitis media occurs by direct spread from the nasopharynx.

Bronchitis: Careful techniques of sputum culture have shown that *H. influenzae* is an important pathogen associated with pneumococci in the acute exacerbations of chronic bronchitis.

Epidemiology: There is considerable similarity between the epidemiology and pathogenicity of *H. influenzae* and of pneumococci. Infection is transmitted by the respiratory route. Carriage in the upper respiratory tract is common particularly in young children, but such strains are usually noncapsulated and not responsible for acute infections.

Immunity is type specific. As the large majority of infections are produced by type *b* strains, the possibility of active immunisation by the specific capsular polysaccharide is under investigation. A type *b* polysaccharide vaccine has been tried, but protection was incomplete in the susceptible group of infants below 18 months, due to poor immunogenicity in some. It may be useful in susceptible adults. Cross reactions have been demonstrated between type *b* polysaccharide and some K antigens of *E. coli*. A strain of *E. coli* 0.75:K100:H5 has been shown to induce antibodies against type *b* polysaccharide when fed to volunteers. But such antibodies have not been shown to be protective.

Laboratory diagnosis: In meningitis, the presence in the CSF of pleomorphic, Gram negative 1
bacilli that do not stain well, should arouse suspicion of *H. influenzae* infection. If the type *b* antiserum is available, the Quellung phenomenon 2
is usually demonstrable as most cases are due to type *b* strains. Capsular polysaccharide antigen 3
may be present in CSF in meningitis and in urine in systemic infections. Its demonstration by precipitation or counterimmunoelectrophoresis with specific antiserum is useful in diagnosis.

For isolation, CSF should be plated promptly on a suitable medium such as blood agar or chocolate agar and incubated in an environment

(H) → humidity

containing 5–10 per cent CO_2 and high humidity. (As the bacillus is very sensitive to low temperatures, specimens should never be refrigerated before inoculation) A strain of staphylococcus should be streaked across the plate. After overnight incubation at 37°C, small opaque colonies appear that show satellitism. Iridescence may be demonstrated on Levinthal's medium. Typing may be done if antisera are available.

Blood cultures are often positive in cases of laryngoepiglottitis and pneumonia. Cultures may be done in nuturient broth as the patient's blood affords sufficient enrichment.

Isolation from sputum requires special care. It has been demonstated that very large variations in the rates of isolation occur when different parts of the sputum are sampled Sputum should, therefore, be homogenised by treatment with pancreatin or by shaking with sterile water and glass beads for 15–30 minutes. Culturing several samples of sputum from the patient increases the rate of isolation.

Treatment: H. influenzae is susceptible to sulphonamides and several antibiotics Chloramphenicol is the drug usually employed for the treatment of haemophilus meningitis, and ampicillin and cotrimoxazole for respiratory infections. Plasmid borne resitance to these drugs is becoming common The newer cephalosporins are the preferred drugs now. Prompt diagnosis and treatment are necessary to avoid serious respiratory complications. Prophylactic rifampin has been recommended for susceptible children in contact with clinical *H. influenzae* infections.

Haemophitus aegypticus
(Koch-Weeks bacillus; H. influenzae biotype III)

Even before Pfeiffer described the 'influenza bacillus', Koch (1883) had observed a small bacillus in conjunctivitis cases in Egypt It was first cultivated by Weeks (1887) in New York. It is worldwide in distribution and causes a highly contagious form of conjunctivitis (pink eye). It is especially common in the tropics and subtropics and may occur in epidemic forms.

H. aegypticus is so closely similar to *H. influenzae* as to be virtually indistinguishable. Conjunctivitis can be produced experimentally by applying the bacillus to the eyes in man, but not in animals. It produces a fatal infection in eight-day-old chick embryos. It does not produce indole.

Haemophitus suis → Swine synd
(H. influenzae suis)

This is a pathogen of swine that aets synergistically with the swine influenza virus. Shope (1951) showed that pigs developed a mild febrile illness when the swine influenza virus was adminstered intranasally. But when *H. suis* also was inoculated with the virus, severe disease resulted He also showed that *H. suis* could be isolated regularly from pigs suffering from swine influenza, but not from healthy animals. It had been suggested that a similar relationship may occur between *H. influenzae* and the human influenza virus, but there has been no evidence for any such association.

H. suis is not pathogenic for man. Capsulated and noncapsulated strains occur, Capsulated strains appear to be antigenically homogeneous and unrelated to *H. influenzae*. Capsulated strains require both X and V factors, while noncapsulated strains require only the X factor.

Haemophilus ducreyi

Ducrey (1890) demonstrated this bacillus in chancroid lesions and, by inoculation into the skin on the forearm, was able to transmit the lesion through several generations.

Chancroid or soft sore is a venereal disease characterised by tender, nonindurated, irregular ulcers on the genitalia. The infection remains localised, spreading only to the regional lymph nodes which are enlarged and painful. Autoinoculation lesions may be produced by contact. There is no immunity following infection, but a

hypersensitivity results, which can be demonstrated by intradermal inoculation of killed bacilli.

H. ducreyi is a short, ovoid bacillus (1–1.5μ x 0.6μ) with a tendency to occur in end-to-end pairs or short chains. It is Gram negative and frequently shows bipolar staining. The microscopic appearance in smears has been described as resembling 'a school of red fish', but the bacilli may often appear Gram positive. It has complex growth requirements. It requires 10 per cent CO_2. Primary isolation is difficult. It can be grown on fresh clotted rabbit blood. Smears made after 24-48 hours' incubation show tangled chains of bacilli. It may also be grown on the chorioallantoic membrane of the chick embryo. On chocolate agar enriched with isovitalex and fetal calf serum, and containing vancomycin as a selective agent, *H. ducreyi* forms small, grey, translucent colonies after incubation at 35°C under 10 per cent CO_2 and high humidity in 2–8 days.

The species is antigenically homogeneous and cultures may be identified by agglutination with the antiserum. Intradermal inoculation of the culture into rabbits produces a local ulcerative lesion.

H. ducreyi is susceptible to sulphonamides and many antibiotics. Cases resistant to sulphonamides and tetracyclines have been reported. Erythromycin or cotrimoxazole is the drug of choice.

Haemophilus parainfluenzae

This differs from *H. influenzae* in requiring the V factor only, and not the X factor. It is a commensal in the upper respiratory tract and has been reported to cause, subacute bacterial endocarditis, 'urethritis' and acute pharyngitis.

Haemophilus haemolyticus

This actively haemolytic species occurs as a commensal in the upper respiratory tract. Colonies on blood agar may be mistaken for those of haemolytic streptococci. It requires both X and V factors. It is not pathogenic. Strains that do not require the X factor have been designated *H. parahaemolyticus*.

Gardnerella vaginalis (Haemophilus vaginalis)

As this bacillus requires neither the X nor V factor, it has been removed from the genus *Haemophilus* and placed in the new genus *Gardnerella*. It is a Gram variable rod, 1–2μ x 0.3–.06 μ in size, non-motile, and noncapsulated. Intracellular granules can be demonstrated by Albert's stain. It grows well on Loeffler's serum in the presence of humidified air and 5% CO_2. For isolation of the organism human blood agar or peptone starch glucose agar containing nalidixic acid, gentamicin and amphotericin are recommended.

It causes nonspecific vaginitis and cervicitis, usually in association with anaerobic bacteria (anaerobic vaginosis), characterised by a malodorous vaginal discharge with high pH (more than 5.0) giving a positive amine test. Gram stained smears show vaginal epithelial cells studded with numerous Gram variable bacteria — the so called 'clue' cells of Gardner and Dukes. Lactobacilli and neutrophil leucocytes are usually absent. Treatment with metronidazole is effective.

Haemophilus aphrophilus

This requires the X factor, but not the V factor. Growth is enhanced by 3–5% CO_2. It has been reported as causing bacterial endocarditis, brain abscesses, sinusitis, pneumonia and abscesses elsewhere. It has been suggested that dogs may act as the source of infection for man. It is related to *Actinobacillus actinomycetem comitans*.

Moraxella lacunata
(Morax-Axenfeld bacillus; Haemophilus duplex)

Morax (1896) and Axenfeld (1897) independently described a short, plump, Gram negative bacillus from subacute conjunctivitis. This was grouped in the genus *Haemophilus* because it

does not grow in ordinary media, but as it does not require the X and V factors, it has been separated into the genus *Moraxella*. Moraxella are parasitic and have their main habitat on the mucosa of nasopharyngeal, conjunctival and genital tracts. They have been classified into six species.

In smears from conjunctival discharges *M. lacunata* appears as short, ovoid (2–3μ x 1μ). Gram negative rods arranged in pairs or short chains. It can be grown in media containing blood, serum or other animal protein. On Loeffler's serum slope, colonies produce indentation or lacunae due to liquefaction (hence the name lacunata). The bacillus is oxidase and catalase positive and does not ferment sugars.

It produces catarrhal conjunctivitis (angular conjunctivitis) that runs a subacute or chronic course. Corneal involvement may occur. A few species of Moraxella occasionally give rise to more severe infections in man such as septic arthritis, meningitis and endocarditis.

M. lacunata is very sensitive to zinc salts and they have a specific effect in the treatment of conjunctival disease. Moraxella are sensitive to penicillin and most other antibiotics.

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37 Bordetella

thumb print appearance' ?

The genus *Bordetello* contains three species, *Bord. pertussis, Bord. poropertussis* and *Bord. bronchiseptico*, which were formerly classified under the genus *Hoemophilus*. As they do not require the X and V factors for growth, they have been separated into a new genus named after Bordet, who along with Gengou, discovered the most important member of this group, *Bord. pertussis*, the causative agent of whooping cough (pertussis). The members of this genus are small, Gram negative coccobacilli that do not ferment carbohydrates and are *strict* aerobes. They are H_2S, indole and VP negative. They are parasitic in the respiratory tract of man or animals and, on primary isolation, grow only on complex media.

Bordet and Gengou (1900) observed a small, ovoid bacillus in the sputum of children suffering from whooping cough and succeeded in cultivating it (1906) in a complex medium. This was soon established as the causative agent of whooping cough. A related bacillus, *Bord. poropertussis* was isolated from mild cases of whooping cough in the United States (1937). This has since been reported from other countries also. *Bord. bronchiseptica* originally isolated from dogs (1911) causes broochopneumonia in animals. It may occasionally infect man, producing a condition resembling pertussis.

Bordetella pertussis

(Bordet-Gengou bacillus; formerly *Haemophilns pertussis*)

Morphology: Bord. pertussis is a small, ovoid coccobacillus (mean length 0.5μ). In primary cultures, cells are of uniform size and shape, but on subculture, they may become longer and thread-like. It is nonmotile and nonsporing. It is capsulated, but tends to lose the capsule on repeated cultivation. The capsule can be demonstrated by special stains, but apparently does not swell in the presence of the antiserum. In culture films, the bacilli tend to be arranged in loose clumps, with clear spaces in-between, giving a 'thumb-print' appearance. Freshly isolated strains of *Bord. pertussis* have fimbriae.

It is Gram negative. Bipolar metachromatic granules may be demonstrated on staining with toluidine blue.

Cultural charocteristics: It is aerobic. No growth occurs anaerobically. It grows best at 35°C – 36°C.

Complex media are necessary for primary isolation. The medium in common use is the Bordet-Gengou glycerine-potato-blood agar. Blood is required apparently not to provide additional nutritive factors, but rather to neutralise inhibitory agents like toxic fatty acids. Charcoal or ion-exchange resins incorporated in culture media may serve the same purpose. After several subcultures, growth may occur on ordinary media like nutrient agar, but such cultures are avirulent.

Growth is slow. After incubation for 48 – 72 hours, colonies on Bordet-Gengou medium are small, dome shaped, smooth, opaque, viscid, greyish white, refractile and glistening, resembling 'bisected pearls' or 'mercury drops'. Colonies are surrounded by a hazy zone of haemolysis.

Confluent growth presents an 'aluminium paint' appearance.

Biochemical reactions: It is biochemically inactive. It does not ferment sugars, form indole, reduce nitrates, utilise citrate or split urea. It produces oxidase and, usually, catalase also.

Resistance: It is a delicate organism, being killed readily by heat (55°C for 30 minutes), drying and disinfectants. But unlike *H. influenzae* it retains viability at low temperatures (0°C – 4°C).

Outside the body, *Bord. pertussis* in dried droplets is said to survive five days on glass, three days on cloth and a few hours on paper.

Antigenic properties: Several antigenic factors have been recognised. On first isolation, all strains appear to be antigenically homogeneous and are agglutinated by a common antiserum.

A heat stable lipopolysaccharide endotoxin can be isolated from the cell wall. Like other endotoxins, it is pyrogenic in animals. It has adjuvant properties, but is not protective. It is common to all smooth strains of *Bord. pertussis*, *Bord. parapertussis* and *Bord. bronchiseptica*.

A heat labile (inactivated at 56°C in 15 minutes) protein toxin has also been identified. It is dermonecrotic on intracutaneous inoculation into mice. Antitoxin appears only after inoculation of extracted toxin (or toxoid), but not after natural infection. It is doubtful whether this toxin plays any pathogenic role in natural infection.

Another heat labile exotoxin called the *pertussis toxin* is considered to be a major virulence factor. It has histamine sensitising and islet cell activating properties. It is considered to be responsible for the paroxysmal cough characteristic of pertussis. Pertussis toxin is present in Phase I, but not in Phase IV strains.

Freshly isolated strains possess heat labile, agglutinating antigens associated with the capsule (K antigens), consisting of different factors, numbered 1 – 14. Factor 7 is common to all strains of the three species of *Bordetella*. Factor 12 is specific for *Bord. bronchiseptica* and Factor 14 for *Bord. parapertussis*. Factors 1 – 6 are found only in strains of *Bord. pertussis*. All strains carry Factor 1 and one or more of the other factors. These antigens are not specific determinants of pathogenicity, but they afford a method of serotyping the strains for epidemiological purposes.

The capsular layer also contains a haemagglutinin, acting best at high temperatures (42°C – 50°C). Red cells from a variety of species are agglutinated. Haemagglutination is specifically neutralised by the antiserum.

Other biologically active substances described include a protein *lymphocytosis promoting factor* which induces pronounced lymphocytosis in mice, a peptide *tracheal cytotoxin* which causes ciliostasis and damage to respiratory epithelial cells, and a *polymorphonuclear leucocyte inhibiting factor*.

Despite the identification of a variety of antigens, their relative roles in pathogenesis and immunity remain ill defined. The biologically active substances determining the virulence of *B. pertussis* appear to be under the control of a single gene.

Variation: *Bord. pertussis* undergoes a smooth to rough variation. All fresh isolates are in the smooth form (Phase I). On subculture, they undergo progressive loss of surface antigens, and pass through phases II and III, finally becoming phase IV, which is the rough, avirulent form.

A reversible change in the capsular antigens has been described as 'modulation'. The bacillus may occur in one of three potential 'modes', X, I and C, each of which has a characteristic surface antigen. Modulation is influenced by the nature of the culture medium. On Bordet-Gengou medium, fresh isolates always occur in the X mode.

Pathogenicity: *Bord. pertussis* is an obligate human parasite, but infection can be produced experimentally in several species of animals, the white mouse being most often employed. Intranasal inoculation in mice induces a charac-

teristic patchy interstitial pneumonia, histologically resembling the human disease. Intraperitoneal inoculation of large doses is fatal, due to toxaemia. Intracerebral inoculation causes a fatal infection. Immunised mice are protected. This forms the basis for the intracerebral mouse potency assay for pertussis vaccines.

In man, after an incubation period of about one to two weeks, the disease takes a protracted course, consisting of three stages, the catarrhal, paroxysmal and convalescent, each lasting approximately two weeks. The onset is insidious, with low grade fever and catarrhal symptoms and a dry, irritating cough. Clinical diagnosis in the catarrhal stage is difficult. This is unfortunate, as this is the stage at which the disease can be arrested by antibiotic treatment. This is also the stage of maximum infectivity. As the catarrhal stage advances to the paroxysmal stage, the cough increases in intensity and comes on in distinctive bouts. During the paroxysm, the patient is subjected to violent spasms of continuous coughing, followed by a long inrush of air into the almost empty lungs with a characteristic 'whoop'. The paroxysmal stage is followed by convalescence, during which the frequency and severity of coughing gradually decrease.

The disease usually lasts 6 – 8 weeks, though in some, it may be very protracted. Complications may be due to: 1) pressure effects during the violent bouts of coughing (subconjunctival haemorrhage, subcutaneous emphysema), 2) respiratory (bronchopneumonia, lung collapse), or 3) neurological (convulsions, coma). Respiratory complications are self-limited, the atelectasis resolving spontaneously, but the neurological complications may result in permanent sequelae such as epilepsy, paralysis, retardation, blindness or deafness.

The infection is limited to the respiratory tract and the bacilli do not invade the bloodstream. In the initial stages, the bacilli are confined to the nasopharynx, trachea and bronchi. Clumps of bacilli may be seen enmeshed in the cilia of the respiratory epithelium. As the disease progresses, inflammation extends into the lungs, producing a diffuse bronchopneumonia with desquamation of the alveolar epithelium.

Blood changes in the disease are distinctive and helpful in diagnosis. A marked leucocytosis occurs, with relative lymphocytosis (total leucocyte counts 20,000 – 30,000 per cmm with 60 – 80 per cent lymphocytes). The erythrocyte sedimentation rate is not increased, except when secondary infection is present.

Epidemiology: Whooping cough is predominantly a pediatric disease, the incidence and mortality being highest in the first year of life. Maternal antibody does not seem to give protection against the disease. Immunisation should, therefore, be started early. The disease is commoner in females than in males at all ages. It is worldwide in distribution. It occurs as epidemics periodically, but the disease is never absent from any community.

The source of infection is the patient in the early stage of the disease. Infection is transmitted by droplets and by fomites contaminated with oropharyngeal secretions. Whooping cough is one of the most infectious of bacterial diseases and nonimmune contacts seldom escape the disease.

The secondary attack rates are highest in close household contacts. Subclinical infections seem to be infrequent, and asymptomatic carriers have not been identified. Natural infection confers protection, though it may not be permanent, and second attacks have been reported.

Bord. pertussis causes 95 per cent of whooping cough cases. About 5 per cent of the cases are caused by *Bord. parapertussis*. This is generally a milder disease and the incidence varies in different countries. About 0.1 per cent of the cases are caused by *Bord. bronchiseptica*. A clinical syndrome resembling whooping cough may also be produced by some other respiratory pathogens, such as adenoviruses and *Mycoplasma pneumoniae*.

Laboratory diagnosis: The bacilli are present in

the upper respiratory tract most abundantly in the early stage of the disease. They may be demonstrated by microscopy or, more reliably, by culture. In the paroxysmal stage, the bacilli are scanty and during convalescence, they are not demonstrable. Antibodies develop late and help only in retrospective diagnosis.

Microscopical diagnosis depends on demonstration of the bacilli in respiratory secretions by the fluorescent antibody technique.

For culture, specimens may be collected by different methods:

1. The cough plate method: Here a culture plate is held about 10 – 15 cm in front of the patient's mouth during a bout of spontaneous or induced coughing, so that droplets of respiratory exudates impinge directly on the medium. This has the advantage that specimens are directly inoculated at the bedside.

2. *The postnasal (peroral) swab:* Secretions from the posterior pharyngeal wall are collected with a cotton swab on a bent wire passed through the mouth. Salivary contamination should be avoided. A West's postnasal swab may be conveniently employed.

3. The pernasal swab: Here a swab on a flexible nichrome wire is passed along the floor of the nasal cavity and material collected from the pharyngeal wall. This method yields the highest percentage of isolations.

The swabs are to be plated without delay. In case of delay, the swab should be transported in 0.25–0.5 ml casamino acid solution, pH 7.2, modified Stuart's medium or Mischulow's charcoal agar. The medium employed is the *glycerine-potato-blood agar of Bordet and Gengou* or one of its modifications. Incorporation of diamidine, fluoride and penicillin (Lacey's DFP medium) makes it more selective. Plates are incubated in high humidity at 35°C–36°C. Colonies appear in 48–72 hours. Identification is confirmed by microscopy and slide agglutination. Immunofluorescence is useful in identifying the bacillus in direct smears of clinical specimens and of cultures. The differentiating features of *Bord. pertussis, Bord. parapertussis* and *Bord. bronchiseptica* are listed in Table 37.1.

For serological diagnosis, rise in titre of antibodies may be demonstrated in paired serum samples by agglutination, gel precipitation or complement fixation tests. As antibodies are late to appear, the second sample of serum should be collected some weeks after the onset of the disease.

Prophylaxis: Preventing the spread of infection by isolation of cases is seldom practicable as infectivity is highest in the earliest stage of the disease when clinical diagnosis is not easy.

TABLE 37.1

Differentiating features of Bordetella species

	Bord. pertussis	*Bord. parapertussis*	*Bord. bronchiseptica*
Cell shape	coccoid	rod	coccoid
Motility	–	–	+
Growth on nutrient agar	–	+	+
Pigment	–	+	–
Nitrate reduction	–	–	+
Urease	–	+	+
Oxidase	+	–	+
Catalase	+	+	+

Specific immunisation with killed *Bord. pertussis* vaccine has been found very effective. It is of the utmost importance to use a smooth phase I strain for vaccine production. The method of inactivation should be such that antigenic potency is unaffected. Detoxication with 0.2% merthiolate during several months' storage at 4°C has been recommended as a satisfactory procedure. It is also perhaps necessary to ensure that the vaccine strain contains a full complement of the serotype antigens. Some purified antigens of the bacillus (e.g., *Pillemer fraction*) have been shown to be protective, but they are not in common use. Alum adsorbed vaccine produces better and more sustained protection and less reaction than plain vaccines. Pertussis vaccine is usually administered in combination with diphtheria and tetanus toxoid (triple vaccine). Not only is this more convenient, but it has also the additional merit that *Bord. pertussis* acts as an adjuvant for the toxoids, producing better antibody response.

In view of the high incidence and severity of the disease in the newborn, it is advisable to start immunisation as early as possible. Three injections at intervals of 4 – 6 weeks are to be given before the age of six months, followed by a booster at the end of the first year of life.

Children under four years who are contacts of the case should receive a booster even if they had been previously immunised. They should also receive chemoprophylaxis with erythromycin. Nonimmunised contacts should receive erythromycin prophylaxis for ten days after contact with the patient has ceased. Pertussis vaccination may induce reactions ranging from local soreness and fever, to shock, convulsions and encephalopathy. Provocation poliomyelitis is a rare complication.

Factors contributing to toxicity or postvaccinal encephalopathy have not been defined. The latter complication is estimated to occur in one in 5 – 10 million injections. Estimated neurological complications of natural disease have ranged from 1.5 to 14 per cent in hospitalised cases; a third of these recover, a third have sequelae and a third die or have severe defects.

If neurological complications develop during DPT immunisation, it should be stopped and the immunisation continued with DT vaccine. To avoid the possibility of complications of pertussis vaccination, an acellular vaccine containing the protective components of the bacillus, first developed in Japan, is being tried.

Treatment: Bord. pertussis is susceptible to several antibiotics (except penicillin), but antimicrobial therapy is beneficial only if initiated within the first ten days of the disease. Erythromycin is the drug of choice. Chloramphenicol, tetracycline and ampicillin are also useful.

Bordetella parapertussis

This is responsible for about five per cent of whooping cough cases and generally produces a mild disease. The characteristics listed in Table 37.1 enable differentiation of this bacillus from *Bord. pertussis*.

Bordetella bronchiseptica
(Bord. bronchicanis)

This is motile by peritrichate flagella. It is antigenically related to *Bord. pertussis* and *Brucella abortus*. It occurs naturally in the respiratory tract of several species of animals. It has been found to cause a very small proportion (0.1 per cent) of cases of whooping cough.

Further Reading

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38 Brucella

The genus *Brucella* consists of very small, non-motile, aerobic, Gram negative coccobacilli that grow poorly on ordinary media and have little or no fermentative powers. They are strict parasites of animals and may also infect man.

Brucellosis is a zoonosis, primarily affecting goats, sheep, cattle, buffaloes, pigs and other animals and transmitted to man by contact with infected animals or through their products. The human disease had been recognised along the Mediterranean littoral from very early times and had been known under various names such as Mediterranean fever, Malta fever and undulant fever. Bruce (1887) isolated a small micro-organism from the spleen of fatal cases in Malta and transmitted the disease to monkeys experimentally. This has been named *Brucella melitensis* (*Brucella* after Bruce; *melitensis* after Melita, the Roman name for Malta). The demonstration by Zammit (1905) that *Br. melitensis* was carried by goats and excreted in their milk led to the virtual elimination of the disease from the British soldiers in Malta by prohibiting the use of goats' milk and milk products. Bang (1897) described *Br. abortus*, the cause of contagious abortion in cattle. The third major species in the genus, *Br. suis* was isolated by Traum (1914) from pigs in the USA. Other species have since been isolated from different animals.

Morphology

Brucellae are coccobacilli or short rods, 0.5–0.7 μ × 0.6–1.5 μ in size, arranged singly or in short chains. The cells are so small that they may be mistaken for cocci, as was done by Bruce who called them *Micrococcus melitensis*. In old cultures irregular forms appear. They are nonmotile, noncapsulated and nonsporing. They are Gram negative and nonacid fast. Bipolar staining is not uncommon.

Cultural characteristics

Brucellae are strict aerobes and do not grow anaerobically. *Br. abortus* is capnophilic, requiring 5–10 per cent CO_2 while *Br. suis* is unaffected. The optimum temperature is 37°C (range 20° – 40°C) and pH 6.6–7.4. They may grow on simple media, though growth is slow and scanty on primary isolation. Growth is improved by addition of serum or liver extract. Liver infusion media were widely used for the cultivation of brucellae. The media employed currently are serum-dextrose-agar, serum-potato-infusion-agar, trypticase soy-agar or tryptose-agar. The addition of bacitracin, polymyxin and cycloheximide to the above media makes them selective.

In liquid media, growth is uniform, and in old cultures a powdery or viscous deposit is formed. On solid media, colonies are small, moist, translucent and glistening. Mucoid, smooth and rough types of colonies appear, associated with changes in antigenic structure and virulence.

They grow in the chorioallantoic membrane of the chick embryos and bring about their death in a few days. All grow intracellulary — *Br. melitensis* in the ectodermal cells and *Br. abortus* and *Br. suis* in cells of mesodermal origin and in the vascular endothelium.

Erythritol has a specially stimulating effect on the growth of most strains of brucella.

Biochemical reactions:

No carbohydrates are ordinarily fermented, though they possess oxidative capacity. They are catalase positive, oxidase positive (except for *Br. neotomae* and *Br. ovis* which are negative) and urease positive. Nitrates are reduced to nitrites. Citrate is not utilised, indole is not produced and MR and VP tests are negative.

Resistance:

Brucellae are destroyed by heat at 60°C in 10 minutes and by 1% phenol is 15 minutes. They are killed by pasteurisation. They may survive in soil and manure for several weeks. They remain viable for 10 days in refrigerated milk, one month in ice cream, four months in butter and for varying periods in cheese depending on its pH. They may also survive for many weeks in meat. They are sensitive to direct sunlight and acid, and tend to die in buttermilk. *Br. melitensis* may remain alive for six days in urine, six weeks in dust and 10 weeks in water.

6 6 10
U D W

Antigenic structure:

The somatic antigens of brucellae contain two main antigenic determinants, A and M which are present in different amounts in the three major species. *Br. abortus* contains about 20 times as much A as M; *Br. melitensis* about 20 times M as A. *Br. suis* has an intermediate antigenic pattern. Absorption of the minor antigenic component from an antiserum will leave most of the major antibody component and such absorbed A and M monospecific sera are useful for species identification by agglutination test. The species identification of brucella strains is not, however, so straightforward and strains are often seen that behave biochemically as *abortus* and serologically as *melitensis* and vice versa. Species and biotype identification depends on a variety of other factors besides antigenic structure.

A large number of antigens have been identified by gel precipitation techniques. Antigenic cross reactions exist between brucellae and *V. cholerae* and persons receiving cholera vaccine may develop brucella agglutinins lasting for about three years. Antigenic cross reactions also exist with *E. coli* 0:116, 0:157; salmonella serotypes group N (0:30 antigen) (Kauffmann and White), *Ps. maltophila*, *Y. enterocolitica* and *F. tularensis*. A superficial L antigen resembling salmonella Vi antigen has been described.

Brucella bacteriophage: Several bacteriophages that lyse brucella strains have been isolated. All these phages are serologically similar. The Tblisi (Tb) phage has been designated as the reference phage and at RTD lyses *Br. abortus* only. *Br. suis* is lysed at 10,000 RTD while *Br. melitensis* is not lysed at all.

Classification

CO_2 Requ H₂S Product

Brucellae may be classified into different species based on CO_2 requirements, H_2S production, sensitivity to dyes (basic fuchsin and thionin), agglutination by monospecific sera, phage lysis and oxidative metabolic tests with amino acids and carbohydrates. The three major species are *Br. melitensis*, *Br. abortus* and *Br. suis*, infecting primarily goats or sheep, cattle and swine, respectively. Three biotypes have been recognised in *Br. melitensis*, 8 (1–9, number 8 deleted) in *Br. abortus* and four in *Br. suis*.

Br. suis strains that produce H_2S are known as 'American' and those that do not, as 'Danish' strains.

Br. neotomae has been isolated from desert wood rats in the USA, *Br. ovis* from sheep in Australia and *Br. canis* from dogs in USA.

Pathogenicity

All the three major species of brucellae are pathogenic to man. *Br. melitensis* is the most

TABLE 38.1

Differential characteristics of Brucella species and biotypes

Species	Biotype	Lysis by phage		CO_2 requirement	H_2S production	Growth on dye media			Agglutination by			Most common host
						Basic Fuchsin 1:50,000	Thionin		Mono-specific sera		Anti-rough serum	
		RTD	RTD x10^4				1:25,000	1:50,000	A	M		
Br. melitensis	1	–	–	–	–	+	–	+	–	+	–	Sheep
	2	–	–	–	–	+	–	+	+	–	–	goats
	3	–	–	–	–	+	–	+	+	+	–	
Br. abortus	1	+	+	±	+	+	–	–	+	–	–	
	2	+	+	+	+	–	–	–	+	–	–	
	3	+	+	±	+	+	+	+	+	–	–	
	4	+	+	±	+	+	–	–	–	+	–	
	5	+	+	–	–	+	–	+	–	+	–	
	6	+	+	–	±	+	–	+	+	–	–	
	7	+	+	–	±	+	–	+	+	+	–	Cattle
	9	+	+	±	+	+	–	+	–	+	–	
Br. suis	1	–	+	–	+	–	+	+	+	–	–	Pigs
	2	–	+	–	–	–	–	+	+	–	–	Pigs, hare
	3	–	+	–	–	+	+	+	+	–	–	Pigs
	4	–	+	–	–	+	+	+	+	+	–	Reindeer
Br. neotomae		–	+	–	+	–	–	–	+	–	–	Wood rat
Br. ovis		–	–	+	–	+	+	+	–	–	+	Sheep
Br. canis		–	–	–	–	–	+	+	–	–	+	Dogs

pathogenic, *Br. abortus* the least and *Br. suis* of intermediate pathogenicity. Human infection may be of three types: 1) Latent infection with only serological, but no clinical evidence, 2) acute or subacute brucellosis, and 3) chronic brucellosis.

Acute brucellosis is known as undulant fever, and is mostly due to *Br. melitensis*. It is associated with prolonged bacteraemia. The fever is irregular, consisting typically of intermittent waves or undulations of pyrexia, of a distinctly remittent type. The symptomatology is varied, consisting of muscular and articular pains, asthmatic attacks, nocturnal drenching sweats, exhaustion, anorexia, constipation, nervous irritability and chills. The usual complications are articular, osseous, visceral or neurological.

Chronic brucellosis, which is usually nonbacteraemic, is a low grade infection, with periodic exacerbations. The symptoms are generally related to a state of hypersensitivity in the patient, the common clinical manifestations being sweating, lassitude and joint pains, with

minimal or no pyrexia. The illness lasts for years.

Brucellosis is primarily a disease of the reticuloendothelial system. Brucellae have a special predilection for intracellular growth and may be demonstrated inside phagocytic cells. This accounts for their refractoriness to chemotherapy and the coexistence of viable bacilli with high levels of circulating antibodies. There is some evidence that cell mediated immunity may play an important role in recovery from brucellosis. This cellular immunity appears to require the cooperation of an unidentified serum factor. The ability of virulent strains of *Br. abortus* to resist the destructive action of phagocytosis is ascribed to the presence of a substance in the cell wall of the bacterium which can be specifically neutralised by antiserum.

The brucellae spread from the initial site of infection through lymphatic channels, to the local lymph glands, in the cells of which they multiply. They then spill over into the bloodstream and are disseminated throughout the body. They have a predilection for the placenta, probably due to the presence in it of erythritol, which has a stimulating effect on brucellae in culture.

Of the laboratory animals, the guinea pig is the most susceptible. The Straus reaction can be elicited in male guinea pigs.

Epidemiology

Human brucellosis results from transmission from animals, directly or indirectly. Man to man transmission does not seem to occur. The animals that commonly act as sources of human infection are goats, sheep, cattle, buffalo and swine. In some parts of the world, infection may also come from dogs, reindeer, caribou, camels and yaks. The modes of infection are by ingestion, contact, inhalation or accidental inoculation.

The most important vehicle of infection is raw milk. Milk products, meat from infected animals and raw vegetables or water supplies contaminated by faeces or the urine of infected animals may also be responsible. Infection by contact occurs when brucellae in vaginal discharges, fetuses, placenta, urine, manure or carcasses enter through skin, mucosa or conjunctiva. Contact infection is especially important as an occupational hazard in veterinarians, butchers and animal handlers, and is particularly common during the calving season. Infection is transmitted by inhalation of dried material of animal origin such as dust from wool. Infection by inhalation is a serious risk in laboratory workers handling brucellae. Infection by accidental inoculation is not infrequent among veterinarians and laboratory workers.

Brucellae have a wide host range, but exhibit a degree of host preference in natural infections— *Br. melitensis* being predominantly in goats and sheep, *Br. abortus* in cattle and *Br. suis* in swine. Foci of infection with brucellae may occur also in wild animal populations independent of domesticated animals. Infection is transmitted among animals directly or through blood sucking arthropods, particularly ticks.

Brucellosis is worldwide in distribution and is endemic in certain areas such as the Mediterranean countries. Human infections are caused by different species of brucellae in different areas. In Great Britain, *Br. abortus* is the only species responsible for human infections, while in the swine rearing areas of the USA, most cases are due to *Br. suis*. Almost all human infections in the different parts of India are due to *Br. melitensis* acquired from goats and sheep. In a serological survey of over 30,000 goats and sheep from Haryana, Mathur (1968) found evidence of infection in about 6.5 per cent of the animals. Of the 88 strains isolated from sheep and goats, 71 were *Br. melitensis* and 17 *Br. abortus*. Cows and buffaloes in rural areas were free from infection, but those in organised farms were found infected. All the isolates from cattle were *Br. abortus*.

Laboratory diagnosis: The clinical manifestations of human brucellosis are variable, and only if a high index of suspicion is maintained will the disease be identified. Clinical diagnosis is often impossible and laboratory aid is, therefore, essential. Laboratory methods for diagnosis

include culture, serology and hypersensitivity tests.

Blood culture is the most definitive method for the diagnosis of brucellosis. Blood is inoculated into a bottle of trypticase-soy broth, tryptose broth or liver infusion broth and incubated at 37°C under 5–10 per cent CO_2. Subcultures are made on solid media every 3–5 days, beginning on the fourth day. Growth may often be delayed and cultures should not be declared negative in less than 4–8 weeks.

The Castaneda method of blood culture has several advantages and is recommended. Here, both liquid and solid media are available in the same bottle. The blood is inoculated into the broth and the bottle incubated in the upright position. For subculture, it is sufficient if the bottle is tilted so that the broth flows over the surface of the agar slant. It is again incubated in the upright position. Colonies appear on the slant. This method minimises materials and manipulation, reducing chances of contamination and risk of infection to laboratory workers.

Fig. 38.1 Castaneda's medium for blood culture

Blood cultures are positive only in about 30–50 per cent of cases, even when repeated samples are tested. *B. melitensis* and *Br. suis* are isolated more readily than *Br. abortus*. Culture may also be obtained from bone marrow, lymph nodes, cerebrospinal fluid, urine and abscesses, if present, and, on occasion, also from sputum, breast milk, vaginal discharges and seminal fluid.

As cultures are often unsuccessful, serological methods are important in diagnosis. Several serological tests have been developed, including agglutination, complement fixation, passive haemagglutination, surface fixation, immunofluorescence and opsonocytophagic tests.

The agglutination test is performed most often. This is a tube agglutination test in which equal volumes of serial dilutions of patient's serum and the standardised antigen (a killed suspension of a standard strain of *Br. abortus*) are mixed and incubated at 37°C for 24 hours. Several sources of error have to be guarded against. Sera often contain 'blocking' or 'nonagglutinating' antibodies. The blocking effect may sometimes be removed by prior heating of the serum at 55°C for 30 minutes or by using 4% saline as the diluent for the test. The most reliable method for obviating the blocking effect and detecting the 'incomplete' antibodies is the antiglobulin (Coombs) test. As prozone phenomenon to high titres (upto 1/640) is very frequent in brucellosis, it is essential that several serum dilutions be tested. A positive agglutination test may be produced by cholera, tularaemia or yersinia infection or immunisation. Cholera induced agglutinins may be differentiated by the agglutinin absorption test and also as they are removed by treatment with 2-mercaptoethanol. In order that results from different laboratories be comparable, it is the practice to express agglutinin titres in International Units. This is done by using a standard reference serum for comparison.

In brucellosis, both IgM and IgG antibodies appear in 7–10 days after the onset of clinical infection. As the disease progresses, IgM antibodies decline, while the IgG antibodies persist or increase in titre. In chronic infections, IgM

may often be absent and only IgG can be demonstrated. The agglutination test identifies mainly the IgM antibody, while both IgM and IgG fix complement. The IgG and IgA antibodies may act as 'blocking' or 'nonagglutinating' antibodies. It is thus evident that the agglutination test is usually positive in acute infection, but may only be weakly positive or even be negative in chronic cases. The results of the agglutination tests, therefore, have to be evaluated carefully. While a high titre of agglutinins, and especially demonstration of a rise in titre, can be taken as diagnostic, even a negative agglutination test may not exclude the possibility of brucellosis.

The complement fixation test is more useful in chronic cases as it detects IgG antibody also. The surface fixation test (Castaneda strip test) is simple and suitable for screening. Its principle is that antibody is able to 'fix' a coloured brucella antigen on a strip of filter paper, preventing its movement along the paper when it is applied over saline. Huddleson's opsonocytophagic test is based on the opsonic activity of brucella antibodies. It is not suitable for diagnostic testing. The indirect immunofluorescence test is a specific and sensitive method for detecting the antibodies and may be positive even when the agglutination test is negative. But the procedure is too complex for routine use. ELISA has been found to be a useful method for screening for antibodies and for differentiation of acute and chronic phase of disease.

Hypersensitivity to brucella antigens may be demonstrated by an intradermal test (brucellin test). This is not useful for the diagnosis of acute infections, but may be helpful in chronic brucellosis, where a positive skin test may sometimes be the only objective indicator of infection. A persistently negative skin test helps to exclude brucellosis. In general, the diagnostic value of brucellin test parallels that of the tuberculin test. 'Brucellergen' the antigen used for the test is commercially available. There is no evidence that antibiotic therapy affects the appearance or persistance of antibodies to brucella organisms.

The methods used for the laboratory diagnosis of human brucellosis may also be employed for the diagnosis of animal infections. In addition, brucellae may be demonstrated microscopically in pathological specimens by suitable staining or by immunofluorescence. Several rapid methods have been employed for detection of brucellosis in herds of cattle. These include the 'rapid plate agglutination test' and the 'Rose Bengal card test'. For detection of infected animals in dairies, pooled milk samples may be tested for bacilli by culture and for antibodies by several techniques. In the 'milk ring test', a sample of whole milk is mixed well with a drop of the stained brucella antigen (a concentrated suspension of killed *Br. abortus* stained with haematoxylin) and incubated in water bath at 70°C for 40–50 minutes. If antibodies are present in the milk, the bacilli are agglutinated and rise with the cream to form a blue ring at the top, leaving the milk unstained. If antibodies are absent, no coloured ring is formed and the milk remains uniformly blue. Whey agglutination test is another useful method for detecting antibodies in milk.

Prophylaxis

As the majority of human infections are acquired by consumption of contaminated milk, prevention consists of checking brucellosis in dairy animals. In many advanced countries, this is achieved by detection of infected animals, their elimination by slaughter and the development of certified brucella-free herds. Pasteurisation of milk is an additional safeguard. Vaccines have been developed for use in animals. These include the living attenuated *Br. abortus* strain 19 vaccine for cattle and the killed H38 adjuvant vaccine or the living attenuated *Br. melitensis* Rev. 1 vaccine for goats and sheep.

A vaccine prepared from *Br. abortus* strain 19-BA has been widely employed for human immunisation in the USSR for protection of persons occupationally exposed to infection. This has not been recommended for use elsewhere.

Treatment

Antibiotic treatment consists in the administration of tetracycline, either alone or along with streptomycin, for a period of not less than three weeks. The response is good in acute infections, but not so satisfactory in chronic cases.

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39 Mycobacterium — I Tuberculosis

Mycobacteria are slender rods that sometimes show branching filamentous forms resembling fungal mycelium. Hence the name 'mycobacteria', meaning fungus-like bacteria. They do not stain readily, but once stained, resist decolourisation with dilute mineral acids. Mycobacteria are therefore called 'acid fast bacilli' or AFB. They are aerobic, nonmotile, noncapsulated and nonsporing. Growth is generally slow. The genus includes obligate parasites, opportunist pathogens and saprophytes.

The first member of this genus to be identified was the lepra bacillus discovered by Hansen in 1868. Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis by satisfying Koch's postulates. Tuberculosis in man was subsequently shown to be caused by two types of the bacillus — the human and bovine types, designated *Mycobacterium tuberculosis* and *M. bovis*, respectively. Three other types of tubercle bacilli were also recognised — the murine type (*M. microti*) from voles, the avain type (*M. avium*) from birds and the cold blooded type (*M. piscium*, *M. marinum*, *M. ranae* and others) from fish, lizard, snakes, frogs and other cold blooded animals. Johne (1895) described *M. paratuberculosis* (Johne's bacillus), the agent causing chronic specific enteritis in cattle. Stefansky (1905) described *M. leprae murium*, the bacillus causing rat leprosy. Mycobacteria were isolated from ulcers, first in Australia (*M. ulcerans*, 1948) and later in Sweden (*M. balnei*, 1954), Uganda (*M. buruli*, 1964) and other places.

Saprophytic mycobacteria were isolated from a number of sources. These included *M. butyricum* from butter, *M. phlei* from grass, *M. stercoris* from dung and *M. smegmatis* from smegma. Several mycobacteria, distinct from human or bovine tubercle bacilli, which have been isolated on occasion from human pathological material, have been grouped together under the loose term 'atypical mycobacteria'. They are also known as 'anonymous' or 'unclassified' mycobacteria. Unlike tubercle bacilli which are strict parasites, atypical mycobacteria may occur in soil, water and other sources.

Mycobacterium tuberculosis

Morphology: M. tuberculosis is a straight or slightly curved rod, 1–4μ × 0.2–0.8 μ occurring singly, in pairs or in small clumps. The size depends on conditions of growth, and long, filamentous, club shaped and branching forms may sometimes be seen. *M. bovis* is usually straighter, stouter and shorter. The bacilli are nonmotile, nonsporing and noncapsulated.

Tubercle bacilli have been described as Gram positive, though strictly speaking they cannot be so described, as after staining with basic dyes, they resist decolorisation by alcohol, even without the mordanting effect of iodine. The Gram positivity is independent of the mordanting effect of iodine and appears to be determined by the same factors as are responsible for acid fastness. When stained by carbol fuchsin by the Ziehl-Neelsen method or by fluorescent dyes (auramine O, rhodamine), they resist decolourisation by 25 per cent sulphuric acid and absolute

TABLE 39 I

Classification of Mycobacteria

A. TUBERCLE BACILLI
  1 Human – *M. tuberculosis*
  2 Bovine – *M. bovis*
  3 Murine – *M microti*
  4 Avian – *M. avium*
  5 Cold blooded – *M marinum*
B. LEPRA BACILLI
  1 Human – *M. leprae*
  2 Rat – *M leprae murium*
C. MYCOBACTERIA CAUSING SKIN ULCERS
  1 *M. ulcerans*
  2 *M. balnei.*
D. ATYPICAL MYCOBACTERIA
  1 Photochromogens
  2 Scotochromogens
  3 Nonphotochromogens
  4 Rapid growers
E. JOHNE'S BACILLUS
  M. paratuberculosis
F SAPROPHYTIC MYCOBACTERIA
  M butyricum, M phlei, M. stercoris, M. smegmatis and others

alcohol for ten minutes (*acid* and *alcohol fast*). Acid fastness has been variously ascribed to the presence in the bacillus of an unsaponifiable wax (mycolic acid) or to a semipermeable membrane around the cell. It is related to the integrity of the cell and appears to be a property of the cell wall. Staining may be uniform or granular. Beaded or barred forms are frequently seen in *M. tuberculosis* while *M. bovis* stains more uniformly.

Nonacid fast rods and granules have been reported in young cultures. Much (1907)[1] demonstrated Gram positive granules in cold abscess pus in which acid fast bacilli could not be found, but which could produce tuberculosis when injected into susceptible animals. Much suggested that these granules (Much's granules) were nonacid fast forms of tubercle bacilli.

Electron micrographs of thin sections show that the thick cell wall is composed of three layers enclosing a plasma membrane which also has got three layers. Spheroplast formation occurs when grown in the presence of lysozyme. L-type growth can also be induced.

Cultural characteristics: The bacilli grow slowly, the generation time *in vitro* being 14–15 hours. Colonies appear only in about two weeks and sometimes may be delayed upto 6–8 weeks. Optimum temperature is 37°C and growth does not occur below 25°C or above 40°C. Optimum pH is 6.4 to 7.0. *M. tuberculosis* is an obligate aerobe while *M. bovis* is microaerophilic on primary isolation, becoming aerobic on subculture. Growth is stimulated by 5–10 per cent CO_2. *M. tuberculosis* grows luxuriantly in culture as compared to *M. bovis* which grows sparsely. They are, therefore, termed 'eugonic' and 'dysgonic', respectively. The addition of glycerol (0 5 per cent) improves the growth of human strains, while it is without effect or may even inhibit bovine strains. Sodium pyruvate improves the growth of both types of bacilli. *M. tuberculosis*

does not grow in media containing 500 mg of P—nitrobenzoic acid, unlike other slow growing nonchromogens.

Tubercle bacilli do not have exacting growth requirements, but are highly susceptible to even traces of toxic substances like *fatty acids* in culture media. The toxicity is neutralised by serum, albumin or charcoal. Several media, both solid and liquid, have been described for the cultivation of tubercle bacilli. The solid media contain egg (Lowenstein-Jensen, Petragnini or Dorset), blood (Tarshis medium), serum (Loeffler's serum slope) or potato (Pawlowsky's). The solid medium most widely employed for routine culture is the Lowenstein-Jensen (LJ) medium without starch, as recommended by the International Union Against Tuberculosis (IUAT). This consists of coagulated hen's egg, mineral salt solution, asparagine and malachite green, the last acting as a selective agent inhibiting other bacteria. A simple medium containing only eggs, malachite green and coconut water has been reported to be a useful and cheap alternative to the Lowenstein-Jensen medium. Among the several liquid media described, Dubos', Middlebrook's, Proskauer and Beck's, Sula's and Sauton's media are the more common. Liquid media are not generally employed for routine cultivation, but are used for sensitivity tests, chemical tests and preparation of antigens and vaccines.

On solid media *M. tuberculosis* forms dry, rough, raised, irregular colonies with a wrinkled surface. They are creamy white initially, becoming yellowish or buff coloured later. They are tenaceous and not easily emulsified. The colonies of *M. bovis* are in comparison, flat, smooth, moist and white breaking up easily when touched.

In liquid media without dispersing agents, the growth begins at the bottom, creeps up the sides and forms a prominent surface pellicle that may extend along the sides above the medium. Diffuse growth is obtained in Dubos' medium containing a detergent Tween-80 (sorbitan monooleate). Virulent strains tend to form long serpentine cords in liquid media, while avirulent strains grow in a more dispersed fashion. The cord factor by itself is not responsible for virulence. The cord factor consists of two mycolic acids linked to a molecule of trehalose (6,6' – dimycolyl – L – D – trehalose). It is present in some nonpathogenic species of mycobacteria as well. Colonial morphology may be modified by the presence of bacteriophage in the strain.

Tubercle bacilli may also be grown in chick embryos and in tissue culture.

Resistance: Mycobacteria are not specially heat resistant, being killed at 60°C in 15–20 minutes. Survival is influenced by the material in which the bacilli are present. Cultures may be killed by exposure to direct sunlight for two hours, but bacilli in sputum may remain alive for 20–30 hours. Bacilli may remain viable in droplet nuclei for 8–10 days. Cultures remain viable for 6–8 months at room temperature and may be stored for two years in the deep freeze cabinet at –20°C

Mycobacteria are relatively resistant to chemical disinfectants, surviving exposure to 5% phenol, 15% sulphuric acid, 3% nitric acid, 5% oxalic acid and 4% sodium hydroxide. It is destroyed by tincture of iodine in five minutes and by 80% ethanol in 2–10 minutes. 80% ethanol has been recommended as a disinfectant for skin, rubber gloves and clinical thermometers. It sterilises pieces of cloth in ten minutes or less.

Biochemical reactions: Several biochemical tests have been described for the identification of mycobacterial species. The more important of them are the following:

1. Niacin test: Human tubercle bacilli form niacin when grown on an egg medium. When 10% cyanogen bromide and 4% aniline in 96% ethanol are added to a suspension of the culture, a canary yellow colour shows a positive reaction. The human bacilli give a positive reaction, while the bovine type is negative. The production of niacin was originally thought to differentiate human strains of *M. tuberculosis* from all other mycobacteria, but it also occurs in *M. simiae* and a few strains of *M. chelonei*.

Aryl ⇒ Atypical

2. Aryl sulphatase test: The enzyme aryl sulphatase is formed by atypical mycobacteria only. The organisms are grown in a medium containing 0.001 M tripotassium phenolphthalien disulphate. 2 N. NaOH is added dropwise to the culture. A pink colour indicates a positive reaction.

3. Neutral red test: Virulent strains of tubercle bacilli are able to bind neutral red in alkaline buffer solution, while avirulent strains are unable to do so.

4. Catalase-peroxidase tests help in differentiating tubercle bacilli from atypical mycobacteria and provide an indication of the sensitivity of the strain to isonicotinic acid hydrazide (INH). Most atypical mycobacteria are strongly catalase positive while tubercle bacilli are weakly positive in comparison. Tubercle bacilli, but not atypical mycobacteria, are peroxidase positive. Catalase and peroxidase activity are lost when tubercle bacilli develop resistance to INH. Catalase negative strains of tubercle bacilli are avirulent for guinea pigs.

A mixture of equal volumes of 30 vol. H_2O_2 and 0.2% catechol in distilled water is added to 5.0 ml of a test culture and allowed to stand a few minutes. Effervescence indicates catalase production and browning of colonies indicates peroxidase activity.

5. Amidase tests: The ability to split amides has been used to differentiate atypical mycobacteria. A useful pattern is provided by using five amides viz. acetamide, benzamide, carbamide, nicotinamide and pyrazinamide. A 0.00164 M solution of the amide is incubated with the bacillary suspension at 37°C and 0.1 ml of $Mn\ SO_4\ 4\ H_2O$. 1.0 ml of phenol solution and 0.5 ml of hypochlorite solution are added. The tubes are placed in boiling water for 20 minutes. A blue colour developing indicates a positive test.

6. Nitrate reduction test: This is positive with *M. tuberculosis* and negative with *M. bovis*.

Mycobacteria secrete a class of chelating agents termed exochelins.

Antigenic properties: Many antigens have been identified in mycobacteria. Group specificity is due to polysaccharide and type specificity to protein antigens. Following infection by tubercle bacilli, delayed hypersensitivity is developed to the protein of the bacillus (tuberculin). Tuberculins from *M. tuberculosis*, *M. bovis* and *M. microti* appear to be indistinguishable. Some degree of antigenic relationship exists between the protein antigens of tubercle bacilli and some atypical mycobacteria, as shown by weak cross reactions in skin testing with different tuberculins. There is also some antigenic relationship between lepra and tubercle bacilli. (A ribonucleoprotein from *M. tuberculosis* reacts with sera from patients with lepromatous leprosy).

By agglutination, agglutinin-absorption, gel precipitation, passive haemagglutination and other serological tests, *M. tuberculosis* strains have been shown to be antigenically homogeneous and very similar to *M. bovis* and *M. microti*, but distinct from other species. Tuberculosis patients possess circulating antibodies against polysacchande, protein and phosphatide antigens of the tubercle bacillus demonstrable by the use of the sensitised erythrocyte, tanned and protein coated erythrocyte, and phosphatide kaolin agglutination tests. These antibodies are of no diagnostic value and appear to be irrelevant in immunity.

Bacteriophage: Many phages have been isolated from natural sources, such as soil and water. Phages have also been isolated from lysogenic strains. Many mycobacteria infected with temperate phages are not truly lysogenic. Instead of being integrated with host chromosome, the phage genome appears free, like a plasmid. This is called pseudolysogeny.

The tubercle bacillus can be classified into four phage types — A, B, C — and a type intermediate between A and B and therefore designated I (for intermediate). Phage Type A is the commonest type and is present worldwide. Type B occurs in Europe and N. America. Type C is seen rarely. Type I is common in India and neighbouring countries.

Phage 33 D isolated from a lysogenic environmental mycobacterium lyses all variants of *M. tuberculosis* but not BCG.

Bacteriocins: *M. tuberculosis* is divisible into two types by means of bacteriocins produced by rapidly growing mycobacteria.

Host range: *M. tuberculosis* causes natural infection in man, other primates, dogs and some other animals which have close contact with man. Experimentally, it is highly infectious for guinea pigs and hamsters, but virtually nonpathogenic for rabbits, cats, goats, bovines and fowls. Mice are moderately susceptible and develop progressive infection following intraperitoneal, intravenous or intracerebral inoculation. Variations in virulence among strains are frequent. Isolates from lupus, scrofuloderma and urogenital tuberculosis are often of low virulence for experimental animals. About two-thirds of the strains isolated from patients in South India exhibit low virulence for guinea pigs.

M. bovis is generally more pathogenic for animals. It produces tuberculosis in cattle, man, other primates, carnivores including dogs and cats, badgers, swine, parrots and possibly some birds of prey. Experimentally, it is highly pathogenic for rabbits, guinea pigs and calves, moderately pathogenic for dogs, cats, horses and rats and nonpathogenic for most fowl.

Though the different types of tubercle bacilli had been given separate species status, it is now generally agreed that at least the mammalian tubercle bacilli should be considered as variants of the single species *M. tuberculosis*. The name 'African type' (sometimes also called *M. africanum*) has been given to a heterogenous group of tubercle bacilli isolated in Africa, that show properties intermediate between human and bovine types. The name 'Asian type' has been used for a variant of human type originally isolated in South India, which possessed low virulence for guinea pigs. They are susceptible to hydrogen peroxide, are isoniazid sensitive and generally belong to phage type I. The Asian type has been isolated from Thailand and Asian expatriates in East Africa. It is sensitive to TCH (Thiophen-2-carboxylic acid hydrazide).

M. avium causes natural tuberculosis in birds and less often in cattle, swine and other animals. It is rarely found in soil or as aetiological agent in human disease. Experimentally it can cause 'Yersin-type' tuberculosis in rabbits and mice — proliferating without producing macroscopic tubercles. It is nonpathogenic for guinea pigs and rats.

M. microti causes natural tuberculosis in voles. It does not infect man naturally, but experimentally it causes local lesions in man, guinea pigs, rabbits and calves

M. microti is antigenically almost identical with the human and bovine types and in its cultural and biochemical properties is intermediate between them.

Pathogenesis: The essential pathology of tuberculosis consists of the production, in infected tissues, of a characteristic lesion, the tubercle. This is an avascular granuloma composed of a central zone containing giant cells, with or without caseation necrosis, surrounded by epithelioid cells and a peripheral zone of lymphocytes and fibroblasts. The tubercle bacillus does not appear to contain or produce a toxin. The basis of the virulence of the bacillus is unknown. The various components of the bacillus have been shown to possess different biological activities which may influence the pathogenesis, allergy and immunity in the disease.

The cell wall of the bacillus induces resistance to infection, causes delayed hypersensitivity, increases reactivity of mice to endotoxin and can replace the whole bacillus in Freund's adjuvant. Tuberculoprotein can elicit the tuberculin reaction and, when bound to a lipid, can induce delayed hypersensitivity. In tissues it induces the formation of monocytes, macrophages, epitheloid cells and giant cells. The bacterial polysaccharide induces immediate hypersensitivity and causes exudation of neutrophils from blood vessels into tissues. Lipids cause the accumulation of macrophage and neutrophils. Phosphatides induce the formation of tubercles consisting of epithelioid cells and giant cells, with some times even caseation.

exudative → an acute infl. rxn → accumulation of oedema fluid, PMN, later on monocytes around the bacilli.
productive → cellular, → enlarge, coalesce, liquefy & undergo caseation

Tuberculous lesions are primarily of two types — exudative and productive. The exudative type is an acute inflammatory reaction with accumulation of oedema fluid, polymorphonuclear leucocytes, and later of monocytes around the bacilli. The lesions may heal by resolution, lead to necrosis of the tissue or develop into productive type. The productive type of lesion is predominantly cellular, composed of a number of tubercles, which may enlarge, coalesce, liquefy and undergo caseation.

The fate of tubercle bacilli entering the body is influenced by a variety of factors such as the dose, virulence and mode of entry of the bacillus, and the age, resistance and hypersensitivity of the host. Tubercle bacilli enter the body commonly by inhalation, less often by ingestion and rarely by inoculation into the skin. When they are inhaled, the bacilli lodge in the pulmonary alveoli, where they are promptly phagocytosed by alveolar macrophages. But instead of being killed, the bacilli multiply intracellularly and eventually disrupt the phagocyte. Phagocytes with ingested bacilli may even act as vehicles transporting the infection to different parts of the body. Intracellular multiplication of the bacillus is interrupted only with the development of specific cellular immunity which sets in about 6–8 weeks after infection.

In children, primary infection leads to the '*primary complex*'. This consists of a subpleural focus of tuberculous pneumonia in the lung parenchyma (Ghon focus) usually found in the lower lobe or the lower part of the upper lobe, together with the enlarged draining lymph nodes. The primary complex is usually an asymptomatic lesion undergoing spontaneous healing, resulting in hypersensitivity to tuberculoprotein (tuberculin allergy) and some degree of specific acquired resistance (immunity). Rarely, the primary infection may lead to haematogenous spread and the development of miliary tuberculosis, meningitis and lesions in different organs such as the spleen, liver and kidneys.

The *adult type* of tuberculosis is generally due to reactivation of the primary infection (post primary progression, endogenous reinfection), or exogenous reinfection. The adult type of pulmonary lesion may heal by resorption, fibrosis and occasionally calcification, or progress to chronic fibrocaseous tuberculosis with tubercle formation, caseation, cavitation and shedding of tubercle bacilli in sputum (*open tuberculosos*). Rarely an acute, rapidly fatal infection may occur in adults.

Epidemiology: Tuberculosis is an ancient disease, evidence of spinal tuberculosis having been discovered in Egyptian mummies. It has been for many centuries the most important of human infections, in its global prevalence, with devastating morbidity and massive mortality. It has been called 'the captain of all the men of death'. Its prevalence increased greatly following the Industrial Revolution, with rapid urbanisation and overcrowding. With improvements in the standards of living, its incidence has come down in the affluent countries. It has been aptly called 'a barometer of social welfare'.

At any given time there are about 20 million open cases of tuberculosis in the world of which 70–80 per cent are in the poor nations. Some three million people die of tuberculosis every year. Between four and five million new cases of open pulmonary tuberculosis arise each year. Without treatment, new bacillary cases remain contagious for about two years before death or partial cure occurs. The national sample survey carried out by the Indian Council of Medical Research (1955–57) indicated a prevalence rate varying from 13 to 25 active cases and 2–8 bacteriologically positive cases per 1000 population. We have in India at present 8–9 million active cases of which 2–2.5 millions are sputum positive and hence infectious, thus constituting a danger to the community. The annual mortality rate from the disease is estimated to be 60–80 per 100,000 of the population.

There is high prevalence of both infection and active disease in the developing countries. Practically everyone is infected by the age of 20 and the infected rates are as high as 10–15 per cent in the first grade of school. Mortality is high in infants

and in children below five years. Between five and 15 years of age, infection is usually asymptomatic and the death rate is very low. Mortality increases after the age of 15 years. The disease is commoner in males than in females, particularly after the age of 35 years. In India, the prevalence in rural areas was found to be not much lower than that in urban parts.

Low socioeconomic status and malnutrition are important predisposing factors. Dusty occupations, especially exposure to silica dust, favour tuberculosis. Doctors, nurses and laboratory workers who have contact with patients and infectious materials are prone to develop the disease. Racial differences in susceptibility have been reported. Negroes and Red Indians have been found to be more susceptible than whites in the USA and the Welsh and Irish than the English, though these differences in susceptibility could be to some extent due to the economic differences between these groups. Hill dwellers who settle in the plains have been reported to be highly susceptible. Tibetan refugees in India had a higher incidence of the disease than the local population.

The major source of infection is the 'open' human case shedding the bacillus in the sputum. The bacilli remain viable for weeks in dust. Inhalation of such dust is the principal mode of infection. The bovine tubercle bacillus used to be responsible for the bulk of intestinal, glandular, bone and joint tuberculosis in the West, infection having been acquired by consumption of milk from infected cattle. Strict control of dairy cattle and of milk has eliminated this danger. In India, human infection with bovine strains is extremely rare. The avian tubercle bacillus may cause human disease very rarely. 'Atypical' mycobacteria may cause human disease simulating tuberculosis. Their importance as human pathogens is increasing in areas where infection with the tubercle bacillus has been controlled.

Laboratory diagnosis: Laboratory diagnosis of tuberculosis may be established by demonstrating the bacillus in the lesions by microscopy, isolating it in culture or transmitting the infection to experimental animals. Demonstration of hypersensitivity to tuberculoprotein is of some diagnostic value, but serological tests are not useful.

Pulmonary tuberculosis

The specimen tested is the sputum. Bacillary shedding is abundant in lesions with caseation and relatively scanty in miliary tuberculosis. The sputum is best collected early in the morning before any meal. If the sputum is scanty, a 24-hour collection may be examined. In early or convalescent cases, bacillary shedding may be intermittent and three consecutive samples should be examined for better results. The sputum should be collected directly into a sterile wide mouthed container free from antiseptics. Disposable waxed cardboard containers are ideal. Where sputum is not available, laryngeal swabs may be examined. In children who tend to swallow the sputum, stomach washings may be tested.

Microscopy: Stained smears are examined directly from the sputum and after concentration. New glass slides should be used for smears and they should not be reused as acid fast bacilli are not always removed from the slides by cleaning. Smears should be made from the thick purulent part of the sputum rather than from the thin watery part. The smears are dried, fixed and stained by the Ziehl-Neelsen technique. The smear is covered with carbol fuchsin and gently heated by steaming, for 5–7 minutes, without letting the stain boil and become dry. The slide is then washed with water and decolorised with 20 per cent sulphuric acid till no more stain comes off and then with 95 per cent ethanol for two minutes. Decolorisation may also be carried out as a single step with acid alcohol (3% HCl in 95% ethanol). After washing, the smear is counterstained with Loeffler's methylene blue, 1% picric acid or 0.2 per cent malachite green for one minute. Under the oil immersion objective, acid fast bacilli are seen as bright red rods while the background is blue, yellow or green depending

on the counterstain used. It has been estimated that at least 10,000 acid fast bacilli should be present per ml of sputum for them to be readily demonstrable in direct smears. A negative report should not be given till at least 100 fields have been examined, taking about ten minutes. A positive report can be given only if two or three typical bacilli have been seen. Smears have been graded depending on the number of bacilli seen: 1+ when 3–9 bacilli are seen in the entire smear, 2+ when 10 or more bacilli are seen in the smear, and 3+ when 10 or more bacilli are seen in most oil immersion fields.

Where several smears are to be examined daily, as in sanatoria, it is more convenient to use fluorescent microscopy. Smears are stained with auramine phenol or auramine rhodamine fluorescent stains and when examined under ultraviolet illumination, the bacilli appear as bright rods against a dark background. Because of the contrast, the bacilli can be readily seen under the high dry objective, enabling large areas of the smear to be examined quickly.

Demonstration of acid fast bacilli microscopically provides only presumptive evidence of tuberculosis as saprophytic mycobacteria may present a similar appearance. Most saprophytic species stain uniformly without any barred or beaded appearance and are usually only acid fast, without being alcohol fast. As saprophytic mycobacteria are present in tap water, rubber tubes, cork or bark, they can get into clinical specimens unless scrupulous care is taken in their collection. Saprophytic bacilli are not present in respiratory secretions, but they are a problem with gastric aspirates, faeces and specimens from the urogenital tract.

Concentration methods: Several methods have been described for the homogenisation and concentration of sputum and other specimens. They may be classified as methods useful for microscopy only and those useful for culture and animal inoculation as well. To the former group belong treatment with antiformin (a mixture of equal parts of liquor soda chlorinata and 15 per cent sodium hydroxide), 0.6 per cent sodium carbonate, sodium hypochlorite (chloros), detergents like tergitol, floatation methods using hydrocarbons, and the autoclave method. These concentration methods kill the bacilli without altering their morphology and staining properties. Methods which concentrate the bacilli without inactivating them and so can be used for culture and animal inoculation, include the following:

Petroff's method: This is one of the simplest methods and probably the most widely employed. The sputum is incubated with an equal volume of 4% sodium hydroxide at 37°C with frequent shaking till it becomes clear, on an average for 20 minutes. It is then centrifuged at 3000 r.p.m. for 30 minutes and the sediment neutralised with N/10 HCl and used for smear, culture and animal inoculation. Excessive exposure to alkali is deleterious and should be avoided.

A simpler method has been described, eliminating centrifugation and neutralisation. Sputum is treated with approximately equal volumes of a sterile solution containing cetrimonium bromide 20g and NaOH 40 g per litre of distilled water. The contents are mixed with a cotton swab and allowed to stand for five minutes. Approximately 0.2 ml of the inoculum is smeared firmly with the swab over the entire surface of acid buffered medium (IUAT-LJ medium with 20.5 g KH_2PO_4 per litre). The same swab is used for inoculating a second slope after stirring the contents again. The results have been found to be as good as Petroff's method.

Instead of alkali, homogenisation can be achieved by treatment with dilute acids (6% sulphuric acid, 3% hydrochloric acid or 5% oxalic acid), N-acetyl cysteine with sodium hydroxide, pancreatin, desogen, zephiran and cetrimide.

Hank's flocculation method, Jungmann's method and treatment with trisodium phosphate are other methods for obtaining material for culture and animal inoculation from sputum samples.

Culture: Cultures are very sensitive for detection

of tubercle bacilli and may be positive with as few as 10–100 bacilli per ml of sputum. The concentrated material is inoculated into at least two bottles of IUAT-LJ medium. If the specimen is positive by microscopy, a direct drug sensitivity test may be set up. Cultures are examined for growth after incubation at 37°C for four days (for rapid growing mycobacteria, fungi and contaminant bacteria) and at least twice weekly thereafter. A negative report is given if no growth appears after 8–12 weeks. Smears from colonies that develop are stained by the Ziehl-Neelsen method and examined. For routine purposes, a slow growing, nonpigmented, niacin positive acid fast bacillus is taken as *M. tuberculosis* (except *M. africanum*). Confirmation is obtained by detailed biochemical studies. When the isolate is niacin negative, identification is made by performing a battery of tests, including growth at 25°C and 45°C, pigment production, animal pathogenicity and biochemical reactions.

Sensitivity tests: As drug resistance is an important problem in tuberculosis, it is desirable that sensitivity test be performed with the isolates as an aid to treatment. Sensitivity tests for tubercle bacilli are mainly of three types. The first is the *absolute concentration method* in which a number of media containing serial concentrations of the drug are inoculated and the minimum inhibitory concentration calculated from the medium with the least drug concentration that inhibits growth. The second is the *resistance ratio method* in which two sets of media containing graded concentrations of the drug are inoculated, one with the test strain and the other with a standard strain of known sensitivity. The third is the *proportion method* which indicates the average sensitivity of a strain, taking into account that any population of tubercle bacilli will contain cells with varying degrees of sensitivity to a drug.

Animal inoculation: The concentrated material is inoculated intramuscularly into the thigh of two healthy guinea pigs about 12 weeks old. Subcutaneous inoculation is not recommended as it leads to a local ulcer, which may be infectious. The animals are weighed before inoculation and at intervals thereafter. Progressive loss of weight is an indication of tuberculosis. One animal is killed at four weeks and if no evidence of tuberculosis is noticed at autopsy, the other is killed after eight weeks. Infected animals give a positive skin test with tuberculin. At autopsy, an infected animal will show the following features:

1. A caseous lesion at the site of inoculation. Sometimes the local lesion may contain pus under tension which may spurt on incision.
2. Enlarged caseous draining lymph nodes. The glands involved may be femoral, inguinal, lumbar, portal, mediastinal and cervical, the severity of lesion diminishing as the distance from the site of inoculation increases.
3. Enlarged spleen showing irregular necrotic areas.
4. Tubercles in the peritoneum.
5. A few tubercles may be seen in the lungs.
6. The kidneys are unaffected.

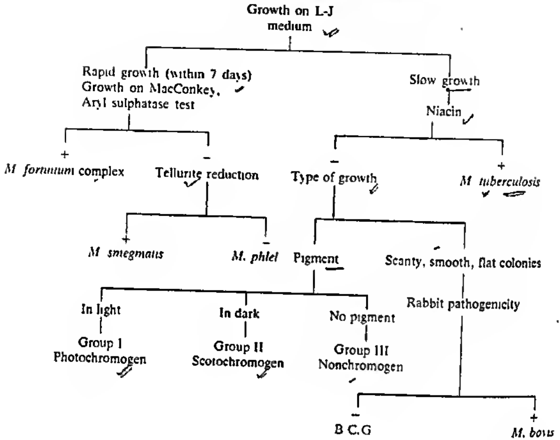
Diagnosis of tuberculosis in the guinea pig has to be confirmed by demonstration of acid fast bacilli in the lesions, as infection with *Y. pseudotuberculosis*, salmonella, brucella, pyogenic cocci and some fungi may simulate the appearance of tuberculosis lesions.

Isolates of *M. tuberculosis* possess varying degrees of virulence for guinea pigs. Catalase negative and INH resistant strains do not produce progressive disease in guinea pigs. Animals inoculated with strains of low virulence may have to be observed for 12 weeks or longer for evidence of infection. Sometimes the only lesion demonstrable may be an enlarged local lymph node. The low virulence of most strains isolated from South India has already been mentioned. For these reasons, guinea pig inoculation is no longer routinely employed for the laboratory diagnosis of pulmonary tuberculosis.

Allergic test: Demonstration of hypersensitivity to tuberculoprotein by the tuberculin test is of limited value only, as it does not differentiate between clinical disease and subclinical infection. It

TABLE 39.2

Identification of tubercle bacilli and related mycobacteria



f some value in indicating active infection in dren below the age of five years. It is also of use reas in which tuberculosis is rare. A negative erculin test often helps to exclude the diag- is of tuberculosis.

ology. Passive haemagglutination and other logical tests may be positive in established 's of tuberculosis, but the results are often tic and usually negative in early cases. No logical test is useful in the diagnosis of tuber- sis.

'apulmonary tuberculosis

uberculous meningitis, the CSF is examined by smear, culture and animal inoculation. On standing, a spider web clot may be formed in the CSF, which contains enmeshed tubercle bacilli. Pleural effusion and other exudates are collected with citrate to prevent coagulation. If free from other bacteria, they are centrifuged and the sediment cultured. If contaminant bacteria are present, the deposit is concentrated before culture. In renal tuberculosis, bacillary shedding may be intermittent. Hence, it is advisable to test 3–6 consecutive morning samples of urine. Each specimen is sent to the laboratory on the day of collection and about 100 ml of it is centrifuged at 3000 r.p.m. for 30 minutes. The deposit is neutralised, if necessary, and stored in the refrigerator. Each day's specimen is treated in this manner and

TABLE 39 3
Features of different strains of tubercle bacilli

Type	Niacin production	Nitrate reduction	Oxygen preference	Growth in TCH medium	Phage type
Classical human	+	+	Aer	+	A.B.C.
Asian	+	+	Aer	−	I
African	+/−	V	M	−	A
Vole	+/−	V	M	−	?
Classical bovine	−	−	M	−	A

V = Variable Aer. = Aerobic M = Microaerophilic
TCH = Thiophen-2-carboxylic acid hydrazide

pooled. The pooled material is concentrated and used for culture and animal inoculation.

Allergy and immunity. Infection with the tubercle bacillus induces delayed hypersensitivity (allergy) and resistance to infection (immunity). The relative importance of allergy and immunity in tuberculosis has long been a matter of controversy, but they represent only different facets of cell mediated immunity which is the only type of immunity operative in tuberculosis. Humoral immunity appears to be of no relevance in tuberculosis and antibodies do not influence the course of the disease. In the nonimmune host, the bacillus is able to multiply inside phagocytes and destroy the cells, while in the immune host the activated T cells release lymphokines which make the macrophages bactericidal. In individuals with defective cell mediated immunity, tuberculosis is refractory to chemotherapy alone. The beneficial effect of the transfer factor in such cases has been established.

The response of a tuberculous animal to reinfection was originally described by Koch. Subcutaneous injection of the virulent tubercle bacillus in a normal guinea pig produces no immediate response but after 10–14 days a nodule develops at the site, which breaks down to form an ulcer that persists till the animal dies of progressive tuberculosis. The draining lymph nodes are enlarged and caseous. If, on the other hand, tubercle bacilli are injected subcutaneously into a guinea pig that had already been infected with tuberculosis 4–6 weeks earlier, an indurated lesion appears at the site in a day or two which undergoes necrosis in another day to form a shallow ulcer that heals rapidly without involvement of the regional lymphatics or other tissues. This is known as the *Koch phenomenon* and is a combination of hypersensitivity and immunity. The Koch phenomenon has three components — a 'local' reaction, a 'focal' response in which there occurs acute congestion and even haemorrhage around tuberculous foci in tissues, and a 'constitutional' or 'systemic' response of fever which may sometimes be fatal.

Allergy can be induced not only by infection with virulent tubercle bacilli but also by injection of attenuated or killed bacilli. Sensitisation with bacillary products is difficult and requires large doses. Injection of tuberculoprotein with a purified wax extracted from the bacillus induces allergy. For the demonstration of allergy, live or killed bacillus or tuberculoprotein (tuberculin) may be employed. Tuberculin allergy is a delayed

1TU = 0.01 mg OT
.00002 mg PPD

hypersensitivity and can be passively transferred by cells but not by serum.

Allergic tests : Koch prepared a protein extract of the tubercle bacillus by concentrating tenfold by evaporation, a 6–8 week culture filtrate of the bacillus grown in 5% glycerol broth. This was called 'Original' or 'Old tuberculin' (OT). Koch first employed OT in the treatment of tuberculosis, but it was soon given up as it was not only not beneficial but also caused serious illness in some patients due to the 'focal' and 'systemic' components of the Koch phenomenon. OT is a crude product and different batches vary in their activity. A purified preparation of the active tuberculoprotein has been standardised by Seibert from cultures grown in semisynthetic medium. This standardised and stable antigen known as Purified Protein Derivative (PPD) is now generally used instead of OT for allergic tests.

The tuberculin test can be done in several ways. Koch originally used subcutaneous injection after which general constitutional symptoms like fever and malaise were taken as indication of sensitivity. This method is still occasionally used in cattle, but is contraindicated in man. Other methods of tuberculin testing not generally used at present are the cutaneous reaction of von Pirquet (rubbing tuberculin into scarified skin), the percutaneous reaction of Moro (rubbing into skin as an ointment), Calmette's ophthalmic reaction after conjunctival instillation, Trambusti's method of introducing into the skin through a wide bore needle puncture, Deane's method of applying in jelly form on abraded skin and the patch test of Vollmer and Goldberger. The tuberculin testing methods in common use now are the graded intradermal test of Mantoux and multiple puncture by the Heaf injector or jet gun.

In the *Mantoux test* which is most widely used, graded doses of tuberculin are injected intradermally on the forearm using a tuberculin syringe. On examination after 48–72 hours, a positive reaction is indicated by oedema and induration at the site, measuring at least 6–10 mm in diameter. Erythema alone is not taken as a positive reaction. The graded doses may range from one tuberculin unit (1 TU, equivalent to 0.01 mg OT or 0.00002 mg PPD) to 100 or 250 TU. All persons with prior infection with the tubercle bacillus, whether clinical or subclinical, exhibit a positive test. Tuberculin allergy wanes gradually and may disappear in four or five years in the absence of subsequent contact with the bacillus. In endemic areas, the allergy is maintained by repeated exposure to the bacillus.

False negative tuberculin tests (anergy) may occur in certain situations such as miliary tuberculosis, convalescence from some viral infections like measles, lymphoreticular malignancy, sarcoidosis, severe malnutrition, immunosuppressive therapy or defective cell mediated immunity. A false positive reaction may be seen in infections by related mycobacteria such as *M. avium* or some 'atypical mycobacteria'. These are usually 'low grade' reactions and can be differentiated by testing with tuberculins prepared from those mycobacteria.

Tuberculin testing may be used as an aid in diagnosing active infection in infants and young children, to measure the prevalence of infection in a community, to select susceptibles for BCG vaccination or as an indication of successful vaccination. Tuberculin testing of cattle has been of great value in the control of bovine tuberculosis.

Prophylaxis. In the prevention of tuberculosis, general measures such as adequate nutrition, good housing and health education are as important as specific antibacterial measures. The latter consist of early detection and treatment of cases, chemoprophylaxis and immunoprophylaxis.

Immunoprophylaxis is by intradermal injection of the live attenuated vaccine introduced by Calmette and Guerin (1921), the Bacille Calmette-Guerin or BCG. This is a strain of *M. bovis* attenuated by 239 serial subcultures in a glycerine-bile-potato medium over a period of 13 years. Injection of BCG in animals leads to dissemination and multiplication of the bacilli in different organs with production of small tubercles. Within a few weeks, however, the bacilli

stop multiplying although they survive in the tissues for an indefinite period of time. The lesions do not spread, but instead disappear slowly. This self-limited infection induces delayed hypersensitivity and immunity. Following BCG vaccination a tuberculin negative recipient is converted to a positive reactor. The immunity has been found to last at least for 10–15 years. The immunity conferred by BCG vaccination is similar to the immunity following natural infection with the tubercle bacillus, except that it does not involve any danger of progressive disease due to reactivation, as in the latter instance

Several field trials have been conducted to assess the efficacy of BCG vaccine (Table 39.4).

The results have varied widely, from 80 per cent efficacy to a total absence of protection. In South India, a trial conducted at Madanapalle showed 60 per cent efficacy, while a recent large trial at Chingleput did not reveal any protection in adults. The reasons for such wide disparity are not clear, but have been attributed to several factors such as differences in the prevalence and virulence of tubercle bacilli in various communities, age and nutritional status of the subjects, type and potency of the vaccines used and the presence of atypical mycobacteria in the areas.

The BCG vaccine had aroused much criticism and it had been suggested that the bacillus may regain its virulence though there has been no evidence of it so far. The Lubeck tragedy in which several children developed fatal tuberculosis following oral BCG vaccination earned the vaccine much notoriety. This was due to an accidental mix-up by which live virulent tubercle bacillus was given instead of the attenuated strain. Stringent safety measures have since been enforced in the manufacture of the BCG vaccine. Considering the millions of doses of the vaccine used, complications have been very few. The complications of BCG vaccination are:

1. *Local:* a) abscess, b) indolent ulcers, c) keloid, d) tuberculides — small satellite BCG tubercles developing in the neighbourhood of the vaccination site, e) confluent reaction in Heaf's multiple puncture technique, f) lupoid lesions, and g) lupus vulgaris.

2. *Regional.* a) enlargement of the draining lymph node, and b) abscess formation in regional lymph nodes.

3. *General* a) fever, b) mediastinal adenitis,

TABLE 39.4
Results of controlled field trials with BCG vaccine

Immunisation Period	Population	Investigators	Observation period (years)	Efficacy of BCG (per cent)
1935-38	North American Indians	Aroson *et al*	18–20	80
1937-48	Chicago infants	Rosenthal *et al*	12–23	74
1947	Georgia school children	Comstock *et al*	20	0
1949-51	Puerto Ricans	Palmer *et al* (U.S.P.H.S.)	5½–7½	31
1950	Georgia/Alabama	Palmer *et al* (U.S.P.H.S.)	14	14
1950-52	British school children (Urban)	M.R.C.	15	79
1950-55	Madanapalle, South India (Rural)	Frimodt-Moller *et al*	9–14	60
1968-71	Chingleput, South India (Rural)	I.C.M.R./W.H.O./ (U.S.P.H.S.)	7½	0

c) erythema nodosum, d) otitis media, e) tendency to keloid formation following wounding at other sites, f) a few cases of nonfatal meningitis have also been reported.

These complications, however, are very rare and respond to appropriate treatment and in no way detract from the benefits of mass vaccination programmes. The handful of cases of progressive tuberculosis reported due to the vaccine strain were probably due to immune deficiency states in the individuals and are more of academic interest.

The consensus of opinion at present is that BCG does protect against tuberculosis, particularly in infants and children. The protection is not absolute, but the disease in the immunised children runs a milder course. It is also believed to prevent skeletal, meningeal and miliary forms of tuberculosis to a large extent. *It is, therefore, recommended that routine BCG immunisation should continue.* Formerly the BCG vaccine was given only to tuberculin negative individuals. This practice continues in the nonendemic advanced countries, but in the developing countries, BCG is offered to all children below the age of 15 years, without prior tuberculin testing (direct BCG). This contributes to more efficient coverage and has been shown to be harmless. BCG is given to infants at birth. This has been found to induce adequate cellular immunity. The vaccine is given intradermally over the deltoid. Freeze dried preparations are employed as they are more stable than the liquid vaccine.

Immunisation with BCG leads to a stimulation of the reticuloendothelial system. It has been reported to confer some protection against leprosy and leukaemia.

Some investigators have found that BCG is superior to PPD for tuberculin testing. It elicits a positive response in a higher proportion of subjects and has the advantage that it also immunises simultaneously.

M. micron, the vole bacillus, has been tried as a live vaccine against tuberculosis. In a controlled trial in Britain, it was found to be as effective as BCG, but as it causes unacceptably severe local reactions its use has been discontinued.

Treatment: Chemotherapy has revolutionised the management of tuberculosis. It has been established that sanatorium regimens, bed rest, fresh air and good food, as well as operative interventions, such as artifical pneumothorax and thoracoplasty, are not essential for cure and that domiciliary treatment with appropriate antibacterial drugs is all that is required. The anti tuberculous drugs employed are rifampicin, isoniazid, pyrazinamide, streptomycin, ethambutol, ethionamide, thiacetazone, paraaminosalycylic acid and cycloserine. The first four of these are bactericidal and the others bacteriostatic. Antituberculous treatment has to be always with multiple drugs — generally, three simultaneously. Prolonged treatment, usually two years or more, used to be the rule. But with the advent of actively bactericidal drugs like rifampicin, short course regimens of about six months have been found adequate in many cases.

The major problem in the chemotherapy of turberculosis is the development of drug resistance. The mechanism of resistance is mutation and selection and hence resistance can be prevented by simultaneous treatment with two or more drugs. Resistance may be 'primary' (pretreatment), or 'secondary' developing during treatment. Inadequate or irresponsible chemotherapy of tuberculosis facilitates the development and dissemination of drug resistant strains, endangering the health of both the patient and the community at large.

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BCG

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40 Mycobacterium — II Atypical Mycobacteria

Mycobacteria other than human or bovine tubercle bacilli, that may occasionally cause human disease resembling tuberculosis, have been called 'atypical', 'anonymous' or 'unclassified' mycobacteria. The name 'opportunist mycobacteria' is better suited as their natural habitat appears to be soil or water and they cause opportunistic infections in man. The name '~~nontuberculous myco-~~bacteria' has gained wide acceptance in recent years. They have also been called 'paratubercle', 'tuberculoid' and 'MOTT' (mycobacteria, other than tubercle) bacilli. There is no evidence of their being transmitted directly from man to man. They are distinct from the saprophytic mycobacteria such as *M. smegmatis* and *M. phlei* which are incapable of infecting man or animals. While human infection with them is common in some areas, disease is rare. They are unable to cause progressive disease when injected into guinea pigs.

Atypical mycobacteria are gaining increasing importance as human pathogens in advanced countries where tuberculosis has been brought under control. Some of them cause pulmonary disease indistinguishable from tuberculosis, while others cause lymphadenitis, urinary infections, cutaneous and subcutaneous lesions. In Britain, atypical mycobacteria are now the commonest cause of cervical adenitis in children. In the developing countries, where tuberculosis is still rampant, atypical mycobacteria are comparatively of minor importance as human pathogens. At the Tuberculosis Research Centre, Madras, of 4943 patients with pulmonary diseases seen over a period of 20 years, only 20 were found infected with atypical mycobacteria, 13 photochromogens and the rest chromogens.

These mycobacteria show a predilection for certain geographical areas, as in South East USA where upto 50 per cent of the population are infected. Such areas are characterised by a high incidence of 'low grade' tuberculin reactions. A general feature of infections with atypical mycobacteria is resistance to antituberculous drugs, particularly streptomycin and INH. Many strains are sensitive to rifampicin. Treatment consists of a combination of drugs selected by sensitivity tests. But chemotherapy is often insufficient and surgery may become necessary.

Atypical mycobacteria have been classified into four groups by Runyon (1959) based on pigment production and rate of growth: Group I Photochromogens, Group II Scotochromogens, Group III Nonphotochromogens and Group IV Rapid Growers. Though other methods of classification have been described, Runyon's classification has found universal acceptance. Species identification depends on several additional characteristics (Table 40.1).

1 *Group I. Photochromogens:* These strains form colonies that produce no pigment in the dark, but when the young culture is exposed to light for one hour in the presence of air, and reincubated for 24–28 hours, a yellow orange pigment appears. They are slow growing, though growth is faster

Not in dark but in light.

TABLE 40 1

Differentiation between tubercle bacilli and some species of atypical mycobacteria

Test	*M. tuberculosis*	*M. bovis*	*M microti*	*M. kansasii*	*M marinum*	*M. scrofulaceum*	*M avium-intracellulare complex*	*M. fortuitum*	*M chelonei*	*M. phlei*	*M smegmatis*
Growth in 7 days	−	−	−	−	−	−	−	+	+	+	+
Growth at 25°C	−	−	−	+	+	+	±	+	+	+	+
Growth at 37°C	+	+	+	+	±	+	+	+	+	+	+
Growth at 45°C	−	−	−	−	−	−	±	−	−	+	+
Pigment in dark	−	−	−	−	−	+	−	−	−	+	−
Pigment in light	−	−	−	+	+	+	−	−	−	+	−
Urease	+	+	+	+	+	+	−	+	+	+	+
Niacin	+	−	±	−	−	−	−	−	−	−	−
Nitrate reduction	+	−	−	+	−	−	−	+	−	+	+

than that of tubercle bacilli. The most important species in this group is *M. kansasii.*

M. kansasii causes chronic pulmonary disease resembling tuberculosis, particularly in old persons with preexisting lung diseases Infections are more common in cities and in industrial areas such as Midwest United States and the mining areas in Wales. Man to man transmission does not seem to occur. The bacilli have been isolated from soil and milk.

Several photochromogenic mycobacteria were isolated in 1964 from monkeys exported from India. They have been classified into two species, niacin positive, *M. simiae* and niacin negative *M. asiaticum.* They have subsequently been associated with pulmonary disease in man.

Group II Scotochromogens: These strains form pigmented colonies (yellow-orange-red) even in the dark. They are widely distributed in the environment and sometimes contaminate cultures of tubercle bacilli They do not usually cause human disease except for *M. scrofulaceum* which may cause scrofula (cervical adenitis) in children.

Indian also

Group III. Nonphotochromogens: These strains do not form pigment even on exposure light. Colonies may resemble those of tubercle bacilli The medically important species are *M. intracellulare, M. avium* and *M xenopi.*

M. intracellulare causes chronic pulmonary disease indistinguishable from tuberculosis. It may also cause renal infection and lymphadenopathy. It is commonly known as the Battey bacillus since it was first identified as a human pathogen at the Battey State Hospital for Tuberculosis in Georgia, USA. Infection with the Battery bacillus is widely prevalent in South East USA and Western Australia. Soil is the probable reservoir. Rifampin with streptomycin is recommended for treatment.

M. avium is so closely similar to the Battey bacillus that they have been considered by some to belong to the same species. It causes tuberculosis in fowls and may also infect pigs. Human infection is rare and may result in cervical adenitis in children or lung disease in the elderly. It is more amenable to chemotherapy than the Battey bacillus.

In view of the many similarities between *M. avium* and *M. intracellulare,* the question whether they are variants of a single species or

separate species is a matter of considerable debate. Temporarily, they are put in a complex along with *M. scrofulaceum* and the whole complex called *M. avium-intracellulare-scrofulaceum complex* or MAIS complex.

The nonphotochromogen *M. malmoense* causes pulmonary disease.

M. xenopi, originally isolated from toads, may occasionally cause chronic lung disease in man. *M. xenopi* and *M. avium* are thermophiles, capable of growth at 45°C.

Though usually classified as a nonphotochromogen, *M. xenopi* may form scotochromogenic yellow colonies. *M. xenopi* has been isolated from water taps, mostly hot water taps, in hospitals. It has also been isolated from main water supplies. Occasionally, it causes renal disease in man.

Group IV. Rapid growers: This is a heterogeneous group of mycobacteria capable of rapid growth, colonies appearing within seven days of incubation at 37°C or 25°C. Within the group, photochromogenic, scotochromogenic, and nonchromogenic species occur. All the chromogenic rapid growers are saprophytes (e.g., *M. smegmatis, M. phlei*). The medically important species are *M. fortuitum* and *M. chelonei* both of which can cause chronic abscesses in man. Outbreaks of abscesses following injection of vaccines and other preparations contaminated by these mycobacteria have been reported on a number of occasions. The bacilli are found in the soil, and infection usually follows some injury.

M. fortuitum and *M. chelonei* do not produce any pigment. Pulmonary lesions caused by *M. fortuitum* cannot be distinguished radiologically from typical tuberculosis. It is resistant to standard antituberculous drugs. No effective chemotherapy is available.

M. szulgai, isolated from pulmonary cavitary lesions, appears to be a photochromogen when grown at 25°C, but a scotochromogen at 37°C. Patients respond to triple drug therapy.

Skin pathogens: Cutaneous lesions may occur in leprosy or tuberculosis, either as localised disease or as part of a generalised infection. In a different class are two species of mycobacteria, *M. ulcerans* and *M. marinum* which are exclusively skin pathogens, causing chronic ulcers and granulomatous lesions on the skin. Systemic invasion does not occur and the regional lymph glands are not involved. Cutaneous localisation is because they multiply optimally at skin temperature.

M. ulcerans: This was originally isolated from human skin lesions in Australia (1948), but has subsequently been recovered from similar lesions from Uganda (Buruli ulcer), Congo, Nigeria,

TABLE 40.2
Differentiation between *M. ulcerans* and *M. marinum*

Character	*M. ulcerans*	*M. marinum*
Distribution	Tropics	Temperate zone
Clinical course	Chronic, progressive ulcer	Self-limited ulcer
Bacilli in ulcer	Abundant	Scanty
Rate of growth	Slower; 4-8 weeks	Faster; 1–2 weeks
Growth at 25°C	—	+
Growth at 37°C	—	+
Culture film	Bacilli in cords	No cord formation
Pigment in light	—	+
Mouse foot pad lesion	Oedema, rarely ulcer	Marked inflammation, purulent ulcer

TABLE 40.3
Atypical mycobacteria associated with human disease

Species	Natural habitat	Type of infection
M. africanum	Animals, soil	Pulmonary.
M. asiaticum	Primates	Pulmonary
M. avium intercellulare	Soil, seawater, animals	Pulmonary, systemic, gastrointestinal, lymphadenitis.
M. chelonei s.s. *chelonei*	Soil, seawater, animals	Porcine heart valves, surgical wound, pulmonary
M. chelonei, s.s. *abscessus*	Soil, seawater, animals	Cutaneous, surgical wound, pulmonary, systemic.
M. fallax	Water, soil	Pulmonary.
M. fortuitum	Water, soil, animals	Pulmonary, surgical wound, cutaneous, systemic, bone and joint.
M. haemophilum	Unknown	Cutaneous, subcutaneous.
M. kansasii	Water, animals	Pulmonary, systemic, skin, joints, lymph nodes
M. malmoense	Unknown	Pulmonary
M. marinum	Aquarium, water, fish	Cutaneous (swimming pool granuloma), joints.
M. scrofulaceum	Soil, water, fomites	Lymphadenitis (usually cervical); pulmonary disseminated.
M. schimoidei	Unknown	Pulmonary.
M. simiae	Primates, water	Pulmonary.
M. szulgai	Unknown	Pulmonary, lymphadenitis, cutaneous, subcutaneous bursitis
M. ulcerans	Unknown	Cutaneous
M. xenopi	Soil, water	Pulmonary, epididymitis

Mexico, Malaysia and New Guinea. Ulcers are usually seen on the legs or arms and are believed to follow infection through minor injuries. After an incubation period of a few weeks, the area becomes indurated and breaks down forming an indolent ulcer which slowly extends under the skin.

Smears from the edge of the ulcer show large clumps of bacilli which are acid fast and alcohol fast. The bacillus grows on Lowenstein-Jensen medium slowly, in 4–8 weeks. The temperature of incubation is critical; growth occurs between 30° and 33°C, but not at 25° or 37°C. Inoculation into the foot pad of mice leads to oedema of the limb, though ulceration is infrequent. A toxin is produced by *M. ulcerans* that causes inflammation and necrosis when injected into the skin of guinea pigs. This is the only known toxin produced by mycobacterium species.

M. marinum: This is a natural pathogen of cold-blooded animals, causing tuberculosis in fish and amphibia. It may also occur as a saprophyte in fresh or salt water. Human infection originates from contaminated swimming pools or fish tanks. The lesion, beginning as a papule and breaking down to form an indolent nucler, usually follows abrasions and therefore occurs on the prominences—elbows, knees, ankles, nose, fingers or toes. It was first described from Sweden under

the name 'swimming pool granuloma', and the bacillus was named *M. balnei* (from *balneum*, meaning bath). It has since been reported from other European countries and from North America. Its distribution is in temperate areas in contrast to *M. ulcerans*, which has a tropical prevalence. Human infection may occur in epidemic form. The ulcers are self-limited and undergo spontaneous healing.

Bacilli are scanty in smears from ulcers. Growth occurs in about two weeks at 30°C (range 25°C–35°C) and primary cultures do not grow at 37°C, but they do so after adaptation. Colonies are nonpigmented in the dark, but become intense orange yellow to red on exposure to light. It is not pathogenic for guinea pigs, but intradermal inoculation in rabbits leads to a superficial granulomatous lesion. Footpad inoculation in mice leads to a more severe lesion than with *M. ulcerans*, local inflammation being followed by a purulent ulcer formation.

Infection with *M. marinum* but not *M. ulcerans* may cause a low grade tuberculin reaction.

M. haemophilum, first described in 1978, causes skin lesions. It requires haemin for growth. It grows at 32°C in 2–4 weeks but not at 37°C.

Table 40.3 shows the range of human infections produced by different species of atypical mycobacteria

Further Reading

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41 Mycobacterium — III Mycobacterium leprae

Leprosy is a disease of great antiquity, having been recognised from *Vedic* times in India and from Biblical times in the Middle East. It probably originated in the tropics and spread to the rest of the world. Leprosy has always been held in superstitious dread and the person suffering from leprosy considered 'unclean' and a social outcaste. The lepra bacillus was first observed by Hansen in 1868. Though this was the first bacterial pathogen of man to be described, it remains one of the least understood. This is because it has not been possible to grow the bacillus in culture media.

Morphology: M. leprae is a straight or slightly curved rod, $1–8\mu \times 0.2–0.5\mu$ in size, showing considerable morphological variations. Polar bodies and other intracellular elements may be present. Clubbed forms, lateral buds or branching may be observed. It is Gram positive and stains more readily than the tubercle bacillus. It is acid fast, but less so than the tubercle bacillus, so that 5% sulphuric acid is employed for decolourisation after staining with carbol fuchsin. It is possible to differentiate between live and dead bacilli in stained smears; the former appear solid and uniformly stained while the latter are fragmented and granular. The percentage of uniformly stained bacilli in tissues (morphological index) provides a method of assessing the progress of patients on chemotherapy and is more meaningful than the old criterion 'bacteriological index' (the number of bacilli in tissues).

The bacilli are seen singly and in groups, intracellularly or lying free outside the cells. They frequently appear as agglomerates, the bacilli being bound together by a lipid-like substance, the glia. These masses are known as 'globi'. The parallel rows of bacilli in the globi present a 'cigar bundle' appearance. In tissue sections, the clumps of bacilli resemble cigarette ends. The globi appear in Virchow's 'lepra cells' or 'foamy cells' which are large undifferentiated histiocytes.

Cultivation: It has not so far been possible to cultivate lepra bacilli either in bacteriological media or in tissue culture. There have been several reports of successful cultivation, but none has been confirmed. One of the best known of such reports came from the Indian Cancer Research Centre, Bombay (1962), where an acid fast bacillus was isolated from leprosy patients, employing human foetal spinal ganglion cell culture. The ICRC bacillus has been adapted for growth on Lowenstein-Jensen medium. Its relation to the lepra bacillus is uncertain.

There have been many attempts to transmit leprosy to experimental animals. But the real break through was the discovery by Shepard (1960) that lepra bacilli could multiply in the foot pads of mice kept at a low temperature (20°C). This observation has been confirmed and has become the standard procedure for experimental work with the bacillus. Following intradermal inoculation into food pads of mice, a granuloma develops at the site in 1–6 months. If cell

mediated immunity is suppressed by thymectomy or administration of antilymphocyte serum, a generalised infection is produced, simulating lepromatous leprosy. The armadillo also has been found susceptible to experimental infection. The nine-banded armadillo (*Dasypus novemcinctus*) is highly susceptible to infection with lepra bacilli. Following inoculation into armadilloes, a generalised infection occurs with extensive multiplication of the bacilli and production of lesions typical of lepromatous leprosy. Some wild caught armadilloes have been observed to be naturally infected with a mycobacterium resembling lepra bacillus. 'Natural disease' has also been identified in the chimpanzee and mangabey monkeys from West Africa, but it is not known whether they have any relevance to human infection.

In animal experiments, the generation time of lepra bacillus has been found to be exceptionally long, 12–13 days on the average but may vary from 8–42 days, in comparison with about 14 hours in the case of the tubercle bacillus and about 20 minutes in the case of coliform bacilli.

Resistance: Lepra bacilli have been found to remain viable in warm humid environment for 9–16 days and in moist soil for 46 days. They survive exposure to direct sunlight for two hours and ultraviolet light for 30 minutes.

LEPROSY

Leprosy is a chronic granulomatous disease of man involving primarily the skin, peripheral nerves and nasal mucosa, but capable of affecting any tissue or organ. The disease may be classified into four types — *lepromatous*, *tuberculoid*, *dimorphous* and *indeterminate*. The type of disease is a reflection of the immune status of the host. It is therefore not permanent and varies with chemotherapy and alterations in host resistance. Bacilli isolated from different types of leprosy do not differ in virulence or other properties.

The two extreme or 'polar' forms of the disease are the lepromatous and tuberculoid types. The lepromatous type is seen where the host resistance is low. The bacilli are seen in large numbers or as globi inside lepra cells or extracellularly. Superficial nodular lesions (lepromata) develop which consist of granulation tissue containing a dense collection of vacuolated cells in different stages of development from mononuclear cells to lepra cells. The nodules ulcerate, become secondarily infected and cause distortion and mutilation. Bacilli invade the mucosa of the nose, mouth and upper respiratory tract and are shed in large numbers in nasal and oral secretions. The reticuloendothelial system, eyes, testes, kidneys and bones are also involved. Bacillaemia is common. The lepromatous type is more infective than the other types. The prognosis is poor. Cell mediated immunity is deficient in lepromatous leprosy and the lepromin test is negative. On the other hand, there is an exaggerated and broad humoral immune response. Antibodies in high titre are seen against mycobacterial as well as several other antigens. Autoantibodies are common. Most cases show biological false positive reaction in standard serological tests for syphilis.

At the other end of the spectrum is tuberculoid leprosy, which is seen in patients with high degree of resistance. The skin lesions are few and sharply demarcated, consisting of macular anaesthetic patches. Neural involvement occurs early and may be pronounced, leading to deformities, particularly in hands and feet. Bacilli are scanty in lesions and infectivity is minimal. Cell mediated immunity is adequate and the lepromin test is positive. Antimycobacterial and autoimmune antibodies are rare. The prognosis is good.

The term borderline or dimorphous type refers to lesions possessing characteristics of both tuberculoid and lepromatous types. It may shift to the lepromatous or tuberculoid part of the spectrum depending on chemotherapy or alterations in host resistance.

The indeterminate type is the early unstable tissue reaction which is not characteristic of either lepromatous or tuberculoid type. In many persons, the indeterminate lesions undergo healing spontaneously. In others, the lesions may progress to tuberculoid or lepromatous types.

Ridley and Jopling have introduced a scale for classifying the spectrum of leprosy into five groups — Tuberculoid (TT), Borderline tuberculoid (BT), Borderline (BB), Borderline lepromatous (BL) and Lepromatous (LL).

Epidemiology: Leprosy is an exclusively human disease and the only source of infection is the patient. The exact mode of infection is not clear. Very large numbers of bacilli are shed in the nasal secretions (approximately 10^7 bacilli per ml) and in discharges from superficial lesions of lepromatous cases. The mode of entry may be either through the respiratory tract or through the skin. Asymptomatic infection appears to be quite common in endemic areas. It is not uncommon for an infectious patient, who appears quite normal and is unaware that he has leprosy, to continue to shed bacilli from his nose and skin for 2–3 years before more obvious signs appear and the disease is recognised. It has been suggested but not proved that insect vectors may have a role in the transmission of leprosy.

Leprosy is not highly communicable. The disease develops in only about five per cent of spouses living with leprosy patients. However, contacts of patients show a high rate of sensitisation to *M. leprae* by lymphocyte transformation tests. The incubation period is very long and averages 2–5 years. It has been estimated to vary from a few months to as long as 30 years. As both the time of exposure and the onset of disease are difficult to identify, estimates of incubation period are only approximations. Exceptionally, the disease may follow a single exposure such as tattooing. It is generally held that intimate and prolonged contact is necessary for infection to take place. The disease is more likely if contact occurs during childhood.

Once worldwide in distribution, leprosy is now confined mainly, but not exclusively, to the underdeveloped areas of the tropics and the Southern hemisphere. An estimated 11 million people suffer from leprosy, of which about four million are in India. Even in endemic countries, the distribution of the disease is patchy and irregular, with areas of high incidence contiguous to areas where it is absent. In South India, for instance, some villages have as many as 50 cases per 1000 population, while the adjoining villages may be virtually free from leprosy. Two main patterns of distribution are met with in endemic areas — the village type and the house type perhaps based on differences in socioeconomic conditions (Fig. 41.1).

Immunity: A high degree of innate immunity against lepra bacilli seems to exist in man so that only a minority of those infected develop clinical disease. Infection with lepra bacilli induces both humoral and cellular immune responses. Humoral antibodies are without deleterious effect on the bacilli while cellular immune mechanisms are capable of destroying them. The type of leprosy in an individual is determined by

TABLE 41.1
Characterisation of the different types of leprosy

	Indeterminate		
	Tuberculoid	*Borderline*	*Lepromatous*
Lepra bacilli in tissues	±	+	+++
Lepromin test	++	±	−
Mycobacterial antibodies	±	+	+++
Lymphocytic infiltration of lesions	+++	+	−
Plasma cells in lymphoid tissue	−	+	+++

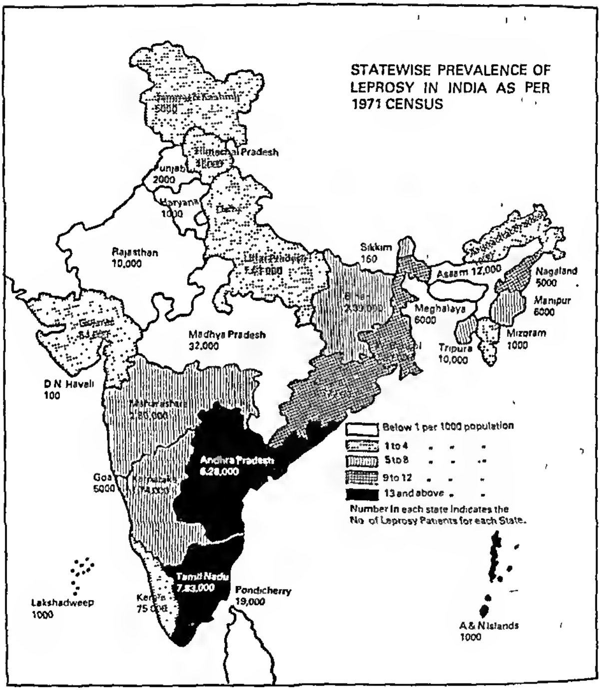

Fig 41.1 Incidence of leprosy in India

the status of cell mediated immunity in him. When it is adequate, the lesions are of the tuberculoid type. The patient exhibits delayed hypersensitivity to the lepra bacillus protein. The macrophages phagocytose the bacilli and destroy them. Specific humoral antibodies are not prominent. There is no increase in the immunoglobulin level and the albumen: globulin ratio in the serum is not altered.

When cell mediated immunity is deficient, the lepromatous type of disease develops. Delayed hypersensitivity to lepra bacillus protein is absent. The macrophages are able to phagocytose the bacilli, but instead of being destroyed, the bacilli proliferate inside the cells. Recent electron microscopic studies suggest that *M. leprae* may evade macrophage antimicrobial activity by escaping from the phagolysosome to lie free in the macrophage cytoplasm. Humoral antibodies are formed in large amounts, consisting of antimycobacterial antibodies and autoimmune antibodies. The albumen:globulin ratio is reversed. The antibodies may possibly have an 'enhancing' effect, antagonising the cellular immunity. A factor having such activity has been identified in the sera of lepromatous patients. Cell mediated immunity is suppressed not specifically against lepra bacillus alone, but in general against other antigens as well. Lepromatous patients are not readily sensitised against such standard antigens as dinitrochlorobenzene and picryl chloride. Transfer factor therapy has been reported to be beneficial.

Though leprosy is a chronic disease, its course is sometimes interspersed with acute exacerbations which are of an allergic nature. Two such reactions are recognised. The 'reversal reaction' is seen in lepromatous cases who develop delayed hypersensitivity and who consequently shift to the borderline or tuberculoid parts of the spectrum. Clinically there is peripheral neuritis, erythema and swelling of skin lesions, which may undergo ulceration. The lesions are infiltrated with small lymphocytes and epithelioid cells, along with a reduction in the numbers of bacilli. The second reaction is 'lepra reaction' or 'erythema nodosum leprosum'. This is usually seen in lepromatous patients undergoing chemotherapy. Clinically, crops of red nodules appear on the skin, lasting for one or two days. The lesions show intense neutrophilic infiltration and the blood vessels in the dermis show acute fibrinoid necrosis. Constitutional disturbances like fever, arthritis, iridocyclitis, orchitis and a painful neuritis are common. The histological picture is that of an Arthus reaction or immune complex disease. Mycobacterial antigens are released into the circulation when the bacilli die and disintegrate during chemotherapy. The combination of these antigens with the precipitating antibodies present in the serum constitutes the mechanism of the lepra reaction.

Lepromin test: Till recently, the only method for studying immunity in leprosy was a skin test for delayed hypersensitivity, the lepromin test first described by Mitsuda in 1919. The original antigen (lepromin) was boiled, emulsified, lepromatous tissue rich in lepra bacilli. The response to the intradermal injection of lepromin is typically biphasic consisting of two separate events. The first is the early reaction of Fernandez, which consists of erythema and induration developing in 24–48 hours and usually remaining for 3–5 days. This is analogous to the tuberculin reaction. Histologically the lesion consists of serous exudate with lymphocytic infiltration. The second is the late reaction of Mitsuda, appearing in three weeks reaching a peak in four weeks and gradually subsiding in the next few weeks. The reaction consists of an indurated skin nodule, which may ulcerate. Histologically, there is infiltration with lymphocytes, epithelioid cells and giant cells. Both the early and late reactions are usually correlated in the same individual, but most leprologists use the late reaction as evidence of lepromin positivity.

Mitsuda's crude antigen is called the integral lepromin. It has been modified in many ways to provide a purer and more standardised antigen, with less of tissue and more of bacillary components. Such partially purified preparations are

called bacillary lepromins. Antigens which consist of the bacillary protein components only are called lepromins and elicit only the early reaction. An antigen commonly employed for lepromin testing is the Dharmendra antigen which is prepared by floating out the bacilli from finely ground lepromatous tissue with chloroform, evaporating it dry and removing the lipids by washing with ether. The antigen is made up in phenol saline for use. Depot lepromin is a modification where the antigen is injected in oily media. Lepromin prepared from lepromatous lesions of armadilloes gives reactions similar to that prepared from human tissue. Armadillo derived lepromin is now widely used.

The lepromin test is not used to diagnose leprosy, nor does it indicate prior contact with the lepra bacillus. Healthy persons in nonendemic areas with no chance of contact with the bacillus may sometimes give a positive lepromin test, probably due to sensitisation by other mycobacterial antigens. The test is employed for the following purposes:

1. To classify the lesions of leprosy patients. The lepromin test is positive in tuberculoid, negative in lepromatous and variable in dimorphous and indeterminate types of disease.

2. To assess the prognosis and response to treatment. A positive reaction indicates good prognosis and a negative one bad prognosis. Conversion to lepromin positivity during treatment is evidence of improvement.

3. To assess the resistance of individuals to leprosy. It is desirable to recruit only lepromin positive persons for work in leprosaria as lepromin negative persons are more prone to develop the disease.

4. To verify the identity of candidate lepra bacilli. Cultivable acid fast bacilli claimed to be lepra bacilli should give matching results when tested in parallel with standard lepromin.

Laboratory diagnosis: Bacteriological diagnosis is easy in the lepromatous but may be difficult in the tuberculoid cases. The diagnosis consists of demonstration of acid fast bacilli in the lesions. For routine examination, specimens are collected from the nasal mucosa, skin lesions and ear lobules. A blunt, narrow scalpel is introduced into the nose and the internal septum scraped sufficiently to remove a piece of mucus membrane, which is transferred to a slide and teased out into a uniform smear. Samples from the skin should be obtained from the edges of the lesion rather than from the centre. The skin is pinched up tight to minimise bleeding and a cut about 5 mm long made with a scalpel, deep enough to get into the infiltrated layers. After wiping off blood or lymph that may have exuded, the scalpel blade is turned transversely to scrape the sides and bottom of the cut so as to obtain a little tissue pulp which is smeared uniformly on a slide. About 5–6 different areas of the skin should be sampled, including the skin over the buttocks, forehead, chin, cheek and ears. The smears are stained by the Ziehl-Neelsen technique using 5% instead of 20% sulphuric acid for decolourisation. Biopsy of the nodular lesions and thickened nerves, and lymph node puncture may be necessary in some cases.

The smears are graded, based on the numbers of bacilli as follows:

1–10 bacilli in 100 fields	= 1+
1–10 bacilli in 10 fields	= 2+
1–10 bacilli per field	= 3+
10–100 bacilli per field	= 4+
100–1000 bacilli per field	= 5+
More than 1000 bacilli, clumps and globi in every field	= 6+

The bacteriological (bacterial) index (BI) is calculated by totalling the number of pluses (+s) scored in all the smears and divided by the number of smears. Thus if eight smears examined have a total of sixteen pluses, the BI will be 2. For calculating BI, a minimum of four skin lesions, a nasal swab and both the ear lobes have to be examined.

The morphological index (MI) is expressed as the percentage of uniformly stained bacilli out of the total number of bacilli counted.

Treatment: Sulphones have been the mainstay in the chemotherapy of leprosy. Diaminodiphenylsulphone (DDS, Dapsone) is administered in gradually increasing amounts till the desired dose is reached and then continued for a number of years. Experiments in mouse footpads have indicated that DDS is effective in doses much smaller than those routinely employed. However, there is evidence that lepra bacilli are becoming resistant to dapsone. Primary resistance has been reported to be as high as 18 per cent in Chinglepattu and 40 per cent in Mali, and secondary resistance, 3.6 per cent in Shanghai and 6 4 per cent in South India. In view of this, multiple drug therapy is now recommended in leprosy, as in tuberculosis. The current recommendation for patients with paucibacillary lesions (I, TT, BT) is the concurrent administration of rifampicin 600 mg once a month and dapsone 100 mg daily for six months. For multibacillary lesions (BB, BL, LL), the recommendation is rifampicin 600 mg once a month, dapsone 100 mg daily and clofazimine 50 mg daily for two years or until skin smears are negative. Ethionamide or prothionamide may be added to this regimen or substituted for clofazimine. A minimum follow up of four years for paucibacillary and eight years for multibacillary cases would be necessary to detect any relapse.

Prophylaxis: Case finding and adequate therapy have been the methods employed for prophylaxis. Long term chemoprophylaxis has given encouraging results in child contacts of infectious cases in India and the Philippines.

There is some degree of antigenic relationship between lepra and tubercle bacilli. It is an old clinical observation that leprosy and tuberculosis do not usually coexist. BCG vaccine was observed to induce lepromin positivity and hence its use in the prevention of leprosy was suggested by Fernandez as early as in 1939. Shepard found that lepra bacilli did not multiply in the footpads of mice immunised with BCG. A controlled trial in Uganda gave very encouraging results, about 85 per cent protection having been obtained against leprosy by BCG vaccination. But another trial in New Guinea was much less encouraging and a trial in Burma did not show any protection More information has to be obtained before mass BCG vaccination can be recommended for the prophylaxis of leprosy.

Attempts are being made to develop a specific vaccine using lepra bacilli grown in armadilloes.

Mycobacterium leprae murium

This is the causative agent of rat leprosy. It was first described by Stefansky in 1901 at Odessa. It has been subsequenttly reported from several countries Rat leprosy is characterised by subcutaneous indurations, lymphadenopathy, emaciation, ulcerations and loss of hair. Acid fast bacilli resembling lepra bacilli are found in the lesions in large numbers. The rat leprosy bacillus does not grow in artifical media but has been grown in tissue cultures. It has a generation time of seven days. It has been suggested that it may be responsible for some cases of human leprosy.

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FA recommended

42 Spirochaetes

Clarification c̄ dr · MCQ.

Elongated, motile, flexible bacteria that are twisted spirally around the long axis are termed spirochaetes (from *speira*, meaning coil and *chaite*, meaning hair). They are structurally more complex than other bacteria. A characteristic feature is the presence of varying numbers of fine fibrils between the outer membrane and the inner peptidoglycan layer of the cell. The fibrils are anchored at the two poles of the cell. The spiral shape and the serpentine motility of the cell depend on the integrity of these filaments. Spirochaetes do not possess flagella, but are motile. Motility is generally of three types — flexion and extension, corkscrew-like rotatory movement and translatory motion. Some are very actively motile while others are sluggish.

Spirochaetes vary widely in size, some being as long as 500μ and others as short as 5μ. Many are free living saprophytes while a few are obligate parasites. They may be aerobic, anaerobic or facultative. Spirochaetes belong to the order *Spirochaetales* which is divided into five genera — *Spirochaeta*, *Cristispira*, *Borrelia*, *Treponema* and *Leptospira*. Members of the genus *Spirochaeta* are free living organisms seen in water and sewage, while *Cristispira* are found in marine and fresh water molluscs.

BORRELIA

Borreliae are large, motile, refractile spirochaetes with irregular, wide and open coils. They are usually 10–30 μ long and 0.3–0.7 μ wide. They are readily stained by ordinary methods and are Gram negative. Several species of borrelia occur as commensals on the buccal and genital mucosa. Borrelia of medical importance are *Borr. recurrentis*, the causative agent of relapsing fever, *Borr. vincentii* which sometimes causes fusospirochaetosis and *Borr. burgdorferi*, responsible for the recently discovered Lyme disease.

Borrelia recurrentis

Relapsing fever has been known since the time of Hippocrates and has occurred in epidemic or sporadic form throughout the world.

The causative borrelia was first observed in the blood of patients by Obermeier and is known as *Borr. recurrentis (obermeirei)*. Borreliae, morphologically indistinguishable, but possessing certain antigenic differences were described by other workers from cases of relapsing fever from different parts of the world. These were given different names, such as *Borr. duttoni* in Central Africa, *Borr. novyi* in America and *Borr. carteri* in India. All of them are now generally considered to be antigenic variants of the single species *Borr. recurrentis*, though some recognise two species based upon differences in the vectors, the borrelia of louse borne relapsing fever being called *Borr. recurrentis* and that of tick borne relapsing fever *Borr. duttoni*.

Morphology: Borr. recurrentis is an irregular spiral with one or both ends pointed. It is 8–20μ long and 0.2–0.4μ wide. It possesses five to ten loose spiral coils at intervals of about 2μ. It stains well with Giemsa and bacterial stains and is Gram negative.

Cultural characteristics: Borrelia are strict anaerobes. Optimum temperature for growth is 28°C – 30°C. Cultivation is difficult, but has been successful in ascitic fluid containing rabbit kidney (Noguchi's medium). Growth occurs on the chorioallantoic membrane of chick embryos. For primary isolation, the best method is to inoculate mice or rats intraperitoneally. When using experimental animals great care has to be taken to ensure that the animals are free from preexisting borreliosis.

Antigenic properties: The borrelia readily undergoes antigenic variations *in vivo* and this is believed to be the reason for the occurrence of relapses in the disease. Ultimate recovery after a number of relapses may be due to the development of immunity to all the antigenic variants. Agglutinating, complement fixing and lytic antibodies develop during infection, but their demonstration is not possible as a routine diagnostic test due to the difficulty in preparing satisfactory antigens.

Pathogenicity: After an incubation period of 2–10 days, relapsing fever sets in as fever of sudden onset. During this period, borreliae are abundant in the patient's blood. The fever subsides after 3–5 days. After an afebrile period of 4–10 days during which borreliae are not demonstrable in blood, another bout of fever sets in. The borreliae reappear in blood during the relapses of fever. The disease ultimately subsides after 3–10 relapses.

Fig. 42.1 *Borrelia recurrentis* in peripheral blood smear

Splenomegaly is common and jaundice occurs in some cases. In fatal cases, necrotic foci containing borreliae in large numbers are seen in the spleen, liver and other organs. Haemorrhagic lesions are seen in the kidneys and intestines. The brain and meninges may also be involved.

Experimentally, rodents such as rats, mice and, less readily, guinea pigs may be infected by intraperitoneal infection. The borreliae may survive in the brain of infected animals after they have disappeared from the blood.

Epidemiology: Relapsing fever may be transmitted by the body louse *(Pediculus corporis)* or by ticks. Louse borne relapsing fever tends to occur as epidemics whenever poverty, overcrowding and lack of personal hygiene encourage louse infestation. Epidemic relapsing fever used to be very common during wars and in jails of former days, but with improvements in hygiene and the discovery of DDT and other insecticides, has become rare. The louse borne disease presents a more severe clinical picture than the tick borne variety and is associated with jaundice, haemorrhages and, in some outbreaks, a high rate of fatality. Infection is transmitted not by the bite of lice, but by their being crushed and rubbed into abraded skin. *Borr. recurrentis* is not transmitted transovarially in lice.

Tick borne relapsing fever occurs as sporadic cases. It is a 'place disease' and is frequently associated with certain dwellings or caves that are inhabited by infected ticks. The disease is milder but relapses are more frequent than in louse borne fever. *Borr. duttoni* persists in the body of infected ticks throughout their life and is also transmitted transovarially so that the ticks act as reservoirs as well as vectors. Infection is transmitted to man through the bite of ticks or through their discharges. Several species of soft ticks belonging to the genus *Ornithodorus* act as vec-

tors, different species being responsible in different regions. In India, the vector species are *O. tholozone, O. crossi, O. lahorensis* and the fowl tick *Argas persicus*. In some areas man is the only mammal infected, but in other areas, rodents and other animals act as the reservoir of infection. Relapsing fever very rarely may be acquired congenitally by transplacental transfer. Laboratory infection may occur through contact with the blood of patients or experimental animals.

Laboratory diagnosis: The borreliae are found in the blood during the fever, but seldom in the apyrexial intervals. A drop of blood may be examined as a wet film under the dark ground or phase contrast microscope and borreliae detected by their lashing movements. Blood smears may be stained with Giemsa or Leishman stain or with dilute carbol fuchsin and examined for borrelia.

A more successful method is to inoculate 1-2 ml of blood from the patient into white mice intraperitoneally The borreliae multiply in the animals and appear in large numbers in peripheral blood within two days. Smears are prepared from blood collected from the tail vein and examined daily for two weeks.

Cultivation of the borreliae and demonstration of antibodies are too difficult and unreliable to be used in diagnosis. Patients with relapsing fever sometimes develop false positive Wassermann reaction. Agglutinins for Proteus OX K are sometimes seen in high titre in louse borne relapsing fever.

Prophylaxis Prevention of louse borne relapsing fever consists of prevention of louse infestation along with the use of insecticides whenever necessary. Prevention of tick borne disease is less easy and consists of identification of tick infested places and their avoidance, or eradication of the vectors No vaccine is available

Treatment: Arsenicals used to he employed formerly, but are now replaced by the antibiotics, penicillin, streptomycin and tetracycline, which are safer and more effective.

Fig 42.2 Throat swab from a case of Vincent's angina showing Vincent's spirochaetes and fusiform bacilli

Borrelia vincenti (*Treponema vincenti*)

Borr. vincenti is a motile spirochaete, about 5-20μ. long and 0.2-0.6μ wide, with three to eight coils of variable size. It is easily stained with dilute carbol fuchsin and is Gram negative. It is a normal mouth commensal but may, under predisposing conditions such as malnutrition or viral infections, give rise to ulcerative gingivostomatitis or oropharyngitis (Vincent's angina). In these cases, *Borr. vincenti* is always associated with fusiform bacilli (*Fusobacterium fusiforme*). This symbiotic infection is known as *fusospirochaetosis*. Large numbers of spirochaetes and fusiform bacilli may also be demonstrated in some cases of lung abscess, phagedenous skin ulcers and gangrenous balanitis. Their significance is uncertain. They are not primary pathogens, but may cause opprotunistic disease in devitalised tissues.

Diagnosis may be made by demonstrating spirochaetes and fusiform bacilli in stained smears of exudates from the lesions. *Borr. vincenti* may be cultivated with difficulty in enriched

media anaerobically. Fusiform bacilli also grow in the culture and it is very difficult to obtain a pure growth. Pencillin and metronidazole are effective in treatment.

Fusospirochaetal infection of the intestine has been reported to cause choleraic diarrohoea or dysentery, but this needs further confirmation.

Borrelia burgdorferi

A new spirochaetal disease identified in 1975 was named *Lyme disease*, as it was first observed in Lyme, Connecticut, USA. The disease has subsequently been reported from other parts of the USA, Europe and Australia. The initial manifestations are an expanding annular skin lesion (erythema chronicum nigrans), fever, headache, myalgia and lymphadenopathy. Weeks or months later, some develop meningoencephalitis, neuropathies and myocarditis. Chronic arthritis is a late sequel.

The causative spirochaete has been named *Borr. burgdorferi*. It can be grown in a modified Kelley's medium, after incubation for two weeks or more. It is sensitive to penicillin and tetracycline. Deer is considered to be the reservoir. The vector is *Ixodes dammini* and related ticks. The spirochaete has been isolated from ticks as well as from skin lesions, CSF and the blood of patients.

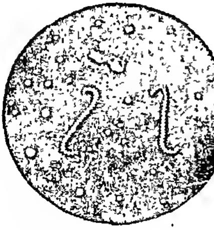

Fig. 42.3 Leptospira — dark ground illumination

LEPTOSPIRA Weil's

Leptospires are actively motile, delicate spirochaetes possessing a large number of closely wound spirals and characteristic hooked ends. They are too thin to be seen under the light microscope (*leptos*, meaning fine or thin). They may be visualised under dark ground illumination. They do not stain readily. Several leptospires are saprophytic while many are parasitic in rodents and other animals. Infection in natural hosts is generally asymptomatic, but when other animals or man are infected, clinical disease may result.

The first recognised leptospiral disease of man was the sprirochaetal jaundice described by Weil (1886). The causative agent of Weil's disease was isolated in 1915 and named *L. icterohaemorrhagiae*. Subsequently a very large number of leptospires have been isolated from human patients and animals from different parts of the world. These were given different names and considered to be different species of leptospires. Several saprophytic leptospires were also isolated from water, sewage and other sources. The genus *leptospira* is now classified into two species *L. interrogans* containing the pathogenic leptospires, and *L. biflexa* containing saprophytic leptospires found predominantly in surface waters. Within each species are serogroups, which are further classified into serotypes (serovars). For example, *L. interrogans* is classified into several serogroups (Icterohaemorrhagiae, Canicola, Pyrogenes, Autumnalis, Australis, Pomona, Hebdomadis, Grippotyphosa, Andamana, etc). Within each serogroup several serovars are recognised, e.g., the serogroup Icterohaemorrhagiae contains the serovars *icterohaemorrhagiae, copenhageni, smithi, budapest*, etc). About 180 serovars have been identified, assembled into 18 serogroups.

Morphology: Leptospires are delicate spirochaetes about 6 – 20 μ long and 0.1 μ thick. They possess numerous coils set so close together that they can be distinguished only under dark ground illumination in the living state or by electron microscopy. Their ends are hooked and resemble

umbrella handles. They are actively motile. They do not take bacterial stains. They may be stained with Giemsa stain. Better results are obtained by silver impregnation methods.

Cultural characteristics: Leprospires can be grown in media enriched with rabbit serum. Several liquid and semisolid media, such as Korthof's, Stuart's and Fletcher's media have been described. They are aerobic and microaerophilic. In semisolid media, growth occurs characteristically a few millimeters below the surface. Optimum temperature is 25°–30°C and optimum pH 7.2–7.5. The generation time in laboratory media is 12–16 hours and 4–8 hours in inoculated animals.

Leptospires may be grown on the chorioallantoic membrane of chick embryos. They may be demonstrated in the blood of allantoic vessels 4–5 days after inoculation. Bacterial contamination is a serious problem in isolating and maintaining leptospires in culture. The use of 5-fluorouracil has been recommended for the inhibition of contaminating bacteria in cultures. A simple method for obtaining cultures free of contaminants is to inoculate the material intraperitoneally in guinea pigs and culture the heart blood collected ten minutes later. Leptospires are able to invade the bloodstream more rapidly than other bacteria.

Resistance. Leptospires are very susceptible to heat, being killed in ten minutes at 50°C and in 10 seconds at 60°C. They are also sensitive to acid and are destroyed by gastric juice in 30 minutes. Bile destroys them rapidly. They are also readily destroyed by most antiseptics and disinfectants. Their survival in water depends on temperature, acidity, salinity and nature and amount of pollution. At 26°C and pH 7.0, they die within 30 days in polluted river water, 18–20 hours in sea water, 12–14 hours in sewage and three minutes in water containing 1 p.p.m. chlorine.

Antigenic properties: Leptospires exhibit considerable antigenic cross reactions. A lipopolysaccharide antigen appears to be present in all members of the genus. Classification into serogroups and serotypes is based on surface antigens, probably composed of protein-polysaccharide complexes. Determination of serotypes is based on agglutination and cross absorption reactions using immune rabbit sera.

Pathogenicity: In natural reservoir hosts, leptospiral infection is asymptomatic. But when infection is transmitted to other animals, clinical disease may result. Man is infected when the leptospires in water contaminated by the urine of carrier animals enter the body through cuts or abrasions on the skin or through intact mucosa of mouth, nose or conjunctiva. The incubation period is usually 6–8 days. The clinical picture varies from mild undifferentiated pyrexia to severe or fatal illness with hepatorenal damage (Weil's disease). In severe cases, the onset is acute, with rigor, vomiting, headache and intense injection of the eyes. The fever is irregular and usually subsides in about ten days. Jaundice occurs in about 10–20 per cent of cases by the second or third day. Purpuric haemorrhages sometimes occur on the skin and mucosa. Albuminuria is a constant feature.

This typical presentation is unusual. Many cases present as aseptic meningitis and in some, abdominal symptoms predominate. Clinical diagnosis is impossible in the majority of cases and unless a high index of suspicion is maintained and laboratory assistance sought, leptospirosis will be missed in all but a few instances.

Leptospires are seen in the blood during the acute phase of the disease, but can seldom be demonstrated after 8–10 days. They persist in the internal organs, and most abundantly in the kidneys, so that they may be demonstrated in the urine in the later stages of the disease.

Serious cases of leptospirosis are caused most often by serotype *icterohaemorrhagiae*, though they may also be due to *copenhageni* and less often *bataviae*, *grippotyphosa*, *pyrogenes*, *andamana* and some others. Aseptic meningitis is common in *canicola* infection and abdominal symptoms in *grippotyphosa* infections.

Laboratory diagnosis: Diagnosis may be made by demonstration of the leptospires microscopically in blood or urine, by isolating them in culture or by inoculation of guinea pigs, or by serological tests.

1. Examination of blood: As leptospires disappear from blood after the first week, blood examination is helpful only in the early stages of the disease, preferably before antibiotics are given. Leptospires may be demonstrated by examination of blood under the dark field microscope or by immunoflourescence. Three or four drops of blood are inoculated into each of several bijou bottles containing Stuart's or Korthof's medium. The bottles are incubated at 37°C for two days and left thereafter at room temperature in the dark for two weeks. Samples from the cultures are examined every third day for the presence of leptospires under dark ground illumination. Chances of isolation are increased by culturing blood daily at the early stage of the disease. Leptospires may sometimes be isolated from CSF also.

The blood from the patient is also inoculated intraperitoneally into young guinea pigs. With virulent serotypes like *icterohaemorrhagiae*, the animals develop fever and die within 8–12 days with jaundice and haemorrhage into the lungs and serous cavities. With other serotypes such as *canicola* and *pomona* the animal may not become ill and infection will have to be identified by demonstration of the leptospires in the peritoneal fluid, by blood culture or by serology. From the third day after inoculation, the peritoneal fluid is examined daily under dark ground illumination and when leptospires are detected, the blood withdrawn by cardiac puncture is inoculated into culture media.

2. Examination of urine: Leptospires appear in the urine in the second week of the disease and intermittently thereafter for 4–6 weeks. The urine should be examined immediately after voiding as leptospires readily undergo lysis in acid urine. Centrifuged deposit of the urine may be examined under dark ground illumination. Direct culture of urine is seldom successful because of contamination, but isolation is usually possible by inoculation into guinea pigs.

The identification of the isolates of leptospires is made by agglutination with type specific sera. Due to the large number of serotypes and the high degree of antigenic cross reactions between them, identification of isolates is a complicated procedure and is generally confirmed by one of the WHO/FAO Reference Laboratories.

3. Serological diagnosis: Antibodies appear in serum towards the end of the first week of the disease and increase till the fourth week, declining thereafter. Agglutinins may, however, be demonstrable years after the infection. Two types of serological tests are available, the broadly reactive screening tests and the serotype specific tests.

The broadly reactive or genus specific tests identify leptospiral infection without indicating the exact infecting serovar. The antigens for these tests are prepared from the nonpathogenic *L. biflexa* Patoc 1 strain. The tests employed include sensitised erythrocyte lysis (SEL), complement fixation, agglutination and indirect immunofluorescence. ELISA has been used to detect IgM and IgG antibodies separately, in order to indicate the stage of infection.

The type specific tests identify the infecting serovar by demonstrating specific antibodies. Macroscopic and microscopic agglutination tests are used for this purpose. In the former, formalinised suspensions of prevalent leptospira serovars are tested for macroscopic agglutination with serial dilutions of the test serum. These antigen kits are available commercially. The microscopic agglutination test generally uses live cultures and agglutination is observed under the low power dark field microscope. This test is more specific and is usually done only in reference laboratories. Due to the presence of some degree of cross reactions between different serovars, agglutination absorption tests may sometimes become necessary for accurate diagnosis.

4. Diagnosis of leptospirosis in animals: Infection in rodents and other animals may be diagnosed by serological tests or by culturing pieces of kidneys.

5. Examination of water for pathogenic leptospires: If a shaved and scarified area of the skin of a young guinea pig is immersed in water for an hour, infection takes place through the abrasions.

Epidemiology: Leptospirosis is a zoonosis. Pathogenic leptospires survive for long periods in the convoluted tubules of the kidneys in natural hosts, multiply and are shed in the urine. Animal carriers often excrete upto 100 million leptospires per ml of urine. If the infected urine contaminates the water or mud that is neutral or slightly alkaline, the leptospires survive for weeks. When man comes into contact with such water, the leptospires enter the body through abraded skin or mucosa and initiate infection. Certain occupational groups such as agricultural workers in rice or cane fields, miners and sewer cleaners are more often exposed to infection, and so leptospirosis is more common in them. Leptospires may be shed in the milk of lactating animals But they die rapidly in milk, and human infection through milk is not known. They are not shed in saliva, and so animal bites are not infectious.

Several animals act as carriers. Rats are particularly important as they are ubiquitous and carry the most pathogenic serotype *icterohaemorrhagiae*. Field mice carry *grippotyphosa*, pigs *pomona* and dogs *canicola* serotypes. But the same serotype may be carried by different mammals and one mammal may carry different serotypes. While leptospires are generally nonpathogenic in the reservoir animal, leptospirosis is of veterinary importance as infection of cattle and pigs cause considerable economic loss. Infection among animals is also transmitted by urinary contamination of water and fodder. Leptospires have been demonstrated in some ticks and nematode parasites of infected mammals, but

TABLE 42.1
Important leptospiral infections

Serotype	*Disease*	*Clinical picture*	*Main source*	*Distribution*
icterohaemorrhagiae	Weil's disease	Fever, jaundice, haemorrhages	Rat	Worldwide
canicola	Canicola fever	Influenza-like, aseptic meningitis	Dog	Worldwide
grippotyphosa	Swamp or marsh fever	Fever, prostration, aseptic meningitis	Field mice	Europe, Africa, S.E. Asia, USA
pomona	Swineherd's disease	Fever	Pig	America, Europe, Middle East, Indonesia, Australia
hebdomadis	Seven day fever Lymphadenopathy	Fever,	Field mice	Japan, Europe, USA
autumnalis	Pretibial fever, Fort Bragg fever.	Fever, rash over tibia	Field mice	Japan, S.E. Asia, USA
pyrogenes	Febrile spirochaetosis	Fever	Field rats	S.E. Asia Europe, USA
bataviae	Indonesian Weil's disease	Fever	Rat	S E. Asia, Africa, Europe
andamana	Weil's disease of Andamans	Fever, jaundice	Rat	S.E. Asia

they do not seem to play any significant role in transmission. Man is an aberrant or 'end' host. There is no evidence that human patients infect others.

Leptospirosis is worldwide in distribution. Certain serotypes such as *icterohaemorrhagiae* are present universally, while others are confined to certain areas. As the facilities for diagnosis of leptospirosis are not widely available, information on the prevalence of the disease is very incomplete.

Prophylaxis: As leptospirosis results from contact of skin or mucosa with contaminated water, general measures of prevention such as rodent control, disinfection of water and the wearing of protective clothing contribute to its prevention. Vaccination has been attempted with some success in dogs, cattle and pigs. Immunity following vaccination or infection is serotype specific. Vaccination has also been tried in persons at high risk such as agricultural workers.

Therapy: Leptospires are sensitive to penicillin and tetracyclines, but treatment to be effective should be started early in the course of the disease. Early serotherapy of Weil's disease, using purified antiserum of high potency has given good results but the serum is not generally available.

TREPONEMA

Treponemes (*trepos*, meaning to turn, and *nema*, meaning thread) are slender spirochaetes with fine spirals and pointed or rounded ends. They can be readily distinguished from borreliae and leptospires morphologically. Some of them are pathogenic for man, while others occur as commensals in the mouth and genitalia. The pathogenic treponemes have not been successfully cultivated, though the commensals may be grown in artificial media. Pathogenic treponemes cause the following diseases in man:

1. Venereal syphilis caused by *T. pallidum*.
2. Endemic syphilis caused by *T. pallidum*.

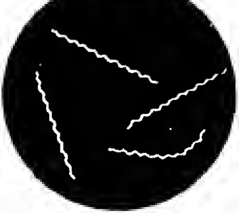

Fig 42.4 *Treponema pallidum* — dark ground illumination

3. Yaws caused by *T. pertenue*.
4. Pinta caused by *T. carateum*.

They are almost identical in their morphology, antigenic structure and other features, though there are differences in the clinical features and natural history of the diseases they produce. It has been suggested that the pathogenic treponemes represent only evolutionary variations of a single species and that the diseases caused by them, though different clinically and epidemiologically, should be considered parts of a continuous spectrum of treponematoses.

Treponema pallidum

Treponema pallidum, the causative agent of syphilis, was discovered by Schaudinn and Hoffmann (1905) in the chancres and inguinal lymph nodes of syphilitic patients. The name pallidum refers to its pale staining.

Morphology: It is a thin, delicate spirochaete with tapering ends, about 10μ in length (range 4–14μ) and 0.1–0.2μ in width. It has about ten regular spirals, which are sharp and angular, at regular intervals of about 1μ. It is actively motile, exhibiting rotation round the long axis, backward and forward movements and flexion of the whole body. During motion, secondary curves appear

and disappear in succession, but the primary spirals are unchanged.

It cannot be seen under the light microscope in wet films but can be made out by negative staining with Indian ink. Its morphology and motility can be seen under the dark ground microscope. It does not take ordinary bacterial stains. It stains light rose red with Giemsa stain. It can be stained by silver impregnation methods. Fontana's method is useful for staining films and Levaditi's for tissue sections.

It is morphologically indistinguishable from *T. pertenue* and some commensal spirochaetes of the mouth and genitalia like *T. microdentium* and *T. mucosum*. Most saprophytic spirochaetes show lashing motility and lack the uniform spirals and their regular spacing at 1μ intervals, as seen in *T. pallidum*.

Cultivation: Beginning with Noguchi (1911), there have been claims of cultivation of *T. pallidum* in artificial media. These have not been confirmed and it is generally agreed that pathogenic treponemes do not grow in artificial media, chick embryos or tissue culture. It is, however, possible to maintain *T. pallidum* in motile and virulent form for 10–12 days in complex media under anaerobic conditions. Nonpathogenic treponemes related to *T. pallidum* (e.g., Reiter strain) are cultivable. Virulent *T. pallidum* can be maintained by serial passage in rabbit testes. One such strain (Nichol's strain) has been maintained for several decades by serial testicular passage since its first isolation in 1912 from the brain of a fatal case of general paralysis of the insane.

Resistance: T. pallidum is very delicate, being readily inactivated by drying or by heat (41° – 42°C in one hour). Hence fomites are of little importance in transmission of infection. Susceptibility of *T. pallidum* to heat was the basis of the 'fever therapy' for syphilis. It is killed in 1–3 days at 0°–4°C, so that transfusion syphilis can be prevented by storing blood for at least four days in the refrigerator before transfusion. Stored frozen at –70°C in 10% glycerol, or in liquid nitrogen (–130°C) it remains viable for 10–15 years. It is inactivated by contact with oxygen, distilled water, soap, arsenicals, mercurials, bismuth, common antiseptic agents and antibiotics.

Antigenic structure: The antigenic structure of *T. pallidum* is poorly understood. Treponemal infection induces at least three types of antibodies. The first is the antibody that reacts in standard or nonspecific serological tests for syphilis, such as Wassermann, Kahn and VDRL, in which a lipid hapten extracted from beef heart is used as the antigen. The hapten is known as cardiolipin and is chemically a diphosphatidyl glycerol. It is not certain whether cardiolipin is an antigen contained in *T. pallidum* itself or whether it is a hapten released from tissues damaged by infection. Antibodies reacting with cardiolipin appear in many other pathological conditions also.

The second antigen is a protein antigen present in *T. pallidum* as well as nonpathogenic treponemes such as the Reiter treponeme. This appears to be a group antigen. Antibodies to this group antigen appear in syphilis and are demonstrable by serological tests in which the Reiter strain is used as the antigen. This antibody is not specific for syphilis and false positive reactions are not uncommon.

The third antigen, probably polysaccharide in nature, is species specific. The antibody to this antigen is demonstrated by the *T. pallidum* immobilisation test which is positive only in the sera of patients infected with pathogenic treponemes.

Pathogenicity: Natural infection with *T. pallidum* occurs only in man. Experimentally, monkeys may be infected. A disease resembling syphilis can be produced experimentally in chimpanzees, with typical lesions of primary and secondary syphilis. Rabbits can be infected by intradermal or intratesticular inoculation, the former giving rise to chancre and the latter to syphilomas. Serial passage in rabbits does not

appear to reduce the virulence of the spirochaete to man, as evidenced by several accidental infections in laboratory workers caused by the Nichol's strain. Hamsters are also susceptible.

SYPHILIS

The origin of syphilis is not definitely known. Towards the end of the fifteenth century, syphilis spread widely throughout Europe in a particularly virulent form. The name 'syphilis' was derived from a poem written by Fracastorius of Verona describing the legend of a shepherd named Syphilus, who had been struck with the disease. It was widely held that syphilis was a new disease brought from America by Columbus's crew. The natural history of the disease has undergone alterations since then, but syphilis continues to be one of the most important and widespread of human infections.

Venereal syphilis is acquired by sexual contact. The spirochaete enters the body through minute abrasions on the skin or mucosa. It multiplies at the site of entry. Clinical disease sets in after an incubation period of about a month (range 10–90 days). The clinical manifestations fall into three stages, primary, secondary and tertiary.

The primary lesion in syphilis is the chancre. In all but a few, the chancre is genital. Other common sites are the mouth and nipples. In some cases the chancre may not be visible, as when it occurs on the uterine cervix. The chancre is a *painless, relatively avascular, circumscribed,* indurated, superficially ulcerated lesion. It is known as 'hard chancre' to distinguish it from the nonindurated lesions of 'soft sore', caused by *H. ducreyi,* and Hunterian chancre after John Hunter who produced the lesion on himself experimentally and described the evolution of the disease. The chancre is covered by a thick, glairy exudate, very rich in spirochaetes. The regional lymph nodes are swollen, discrete, rubbery and nontender. Even before the chancre appears, the spirochaetes spread from the site of entry into the lymph and bloodstream, so that the patient may be infectious during the late incubation period. The chancre invariably heals in about 10–40 days, even without treatment, leaving a thin scar.

Secondary syphilis sets in two to six months after the primary lesion heals, during which period the patient is asymptomatic. The secondary lesions are due to widespread multiplication of the spirochaetes and their dissemination through the blood. Roseolar or papular skin rashes, mucous patches in the oropharynx and condylomata at mucocutaneous junctions are the characteristic lesions. Spirochaetes are abundant in the lesions and consequently the patient is most infectious during the secondary stage. There may also be ophthalmic, osseous and meningeal involvement. Secondary lesions are highly variable in distribution, intensity and duration, but they usually undergo spontaneous healing, in some instances taking as long as four or five years.

After the secondary lesions disappear, there is a period of quiescence known as 'latent syphilis'. Diagnosis during this period is possible only by serological tests. In many cases, this is followed by natural cure but in others, after several years, manifestations of tertiary syphilis appear. These consist of cardiovascular lesions including aneurysms, chronic granulomata (gummata) and meningovascular manifestations. Tertiary lesions contain few spirochaetes and may represent manifestations of delayed hypersensitivity. In a few cases, neurological manifestations such as *tabes dorsalis or general paralysis of the insane develop* several decades after the initial infection. These are known as late tertiary or quaternary syphilis.

In syphilis acquired nonvenereally (as occupationally in doctors or nurses), the natural evolution is as in venereal syphilis, except that the primary chancre is extragenital, usually on the fingers. In the rare instances where syphilis is transmitted by blood transfusion, the primary chancre does not occur. In congenital syphilis, where infection is transmitted from mother to offspring transplacentally, the manifestations and course are different. The obstetric history in a syphilitic woman is one of abortions and

stillbirths followed by live births of infants with stigmata of syphilis and finally of healthy infants.

Laboratory diagnosis: As the manifestations of syphilis are protean and as there are asymptomatic periods during the natural course of the disease, laboratory aid is essential for the diagnosis of the disease. It is also important in assessing the cure after treatment. Because of the social and emotional overtones of the disease, the diagnosis of syphilis should impose a great sense of responsibility on the laboratory. Laboratory diagnosis consists of demonstration of the spirochaetes under the microscope and of antibodies in serum or CSF.

Diagnosis by microscopy is applicable in primary and secondary stages and in cases of congenital syphilis with superficial lesions. Specimens should be collected with care as the lesions are highly infectious. The lesion is cleaned with a gauze soaked in warm saline and the margins gently scraped so that the superficial epithelium is abraded. Gentle pressure is applied to the base of the lesion and the serum that exudes is collected preventing admixture with blood. Wet films are prepared with the exudate and after applying thin coverslips, examined under the dark ground microscope. *T. pallidum* is identified by its slender spiral structure and slow movement. Differentiation from saprophytic spirochaetes commonly present in the genital area needs experience. If antiseptics have been applied to the lesion, it should be cleaned well and a wet saline dressing applied for a day before spirochaetes are looked for. Local application of penicillin diminishes the chances of finding spirochaetes. If the chancre is healing, spirochaetes may not be demonstrable in it, but examination of fluid aspirated from the regional lymph node may be positive. If examination cannot be done immediately, the exudate should be collected in capillary tubes, the ends sealed and transported to the laboratory.

Dark ground examination is useful, but negative results do not exclude the diagnosis of syphilis. Repeated examinations are sometimes necessary. A fluorescent antibody test on the smears of the exudate gives a higher positive rate.

Serological tests: These tests form the mainstay of laboratory diagnosis. There are a bewildering array of tests available, varying in their sensitivity and specificity. They may be classified, depending on the antigens used, into the following:

1. Standard tests for syphilis (STS) in which lipoidal or cardiolipin antigens are used.

2. Treponemal tests in which treponemes are used as the antigen. These are of two kinds: a) those using cultivable treponemes, such as Reiter strain, as the antigen, and b) those in which pathogenic *T. pallidum* (Nichol's strain) is the antigen employed. Treponemal tests may also be classified according to whether the antigen employed is live, killed or extracts of treponemes.

Standard tests for syphilis: Wassermann, Neisser and Bruck (1906) devised the complement fixation test for syphilis using as antigen, a watery extract of the liver of a syphilitic fetus. They used syphilitic liver as a source of treponemes and believed that the test was specific, but Marie and Levaditi (1907) found the extracts of normal liver gave identical results. It was subsequently found that better results were obtained by using an alcoholic extract of ox heart tissue, to which lecithin and cholesterol were added. This crude antigen was standardised by Pangborn (1942-45) who introduced cardiolipin, a purified extract of beef heart.

The standard tests that are usually employed are Wassermann, Kahn and the Venereal Diseases Research Laboratory (VDRL) tests. The Wassermann reaction is a complement fixation test. The patient's serum is 'inactivated' by heating at 56°C for 30 minutes to destroy complement. It is then incubated with cardiolipin antigen and guinea pig complement. If the serum contains antibody to cardiolipin, the added complement is used up in the antigen-antibody reaction. If antibodies are absent, the complement is left behind. So the next step is to test for the pre-

sense or absence of complement in the system. This is done by adding an indicator, haemolytic system consisting of sheep erythrocytes and antisheep erthrocyte antiserum prepared by immunisation of rabbits (amboceptor). The amboceptor causes lysis of the erythrocytes if complement is available. So, if haemolysis occurs after incubation with the haemolytic system, it indicates that the complement was not utilised in the primary reaction because antibody to cardiolipin was absent. This is the case when the patient is not syphilitic, i.e., the test is negative. If, on the other hand, haemolysis does not occur, it indicates that complement was not available as it had been utilised in the primary reaction between cardiolipin and antibody. This is the positive test. The Wassermann reaction was the earliest and, for a long time, the most popular serological test for syphilis, but because of its complexity, it has been largely supplanted by the simpler tests like Kahn or VDRL.

The Kahn test is a tube flocculation test. In this test, 0.15 ml serum taken in three tubes is mixed with different amounts (0.05, 0.025, 0.0125 ml) of freshly prepared antigen dilution, shaken in the Kahn shaker at 280 oscillations per minute and examined after addition of saline. The negative test shows uniform opalescence. Floccules appear in the positive test. The result is graded depending on the appearance of floccules in the three tubes.

With the recognition that false positive reactions were encountered, an attempt was made to make the test more specific by developing the Kahn verification test. Here the test is observed both at 1°C and 37°C. The antibody due to syphilis generally gives a stronger reaction at 37°C and the nonspecific antibody at 1°. This modification has not succeeded in obviating false positive reactions.

The VDRL test has several advantages over the Wassermann and Kahn tests and, therefore, is today the most widely used serological test for syphilis. It is a simple and rapid test which requires only a small quantity of serum and is as sensitive and no less specific than the other tests. In the slide flocculation test which is in routine use, 0.05 ml of inactivated serum is taken in special slides with depressions. Alternatively, slides may be prepared by application of paraffin rings. One drop of the freshly prepared antigen is added with a syringe delivering 60 drops to the ml. The slide is rotated at 180 rotations per minute in a VDRL rotator for four minutes. It is then examined under the microscope with the low power objective. Uniform distribution of crystals in the drop indicates the serum is nonreactive, while the formation of clumps indicates that it is reactive. With reactive sera, serial dilutions can be tested to obtain the reactive titre. Sera containing high titres of antibody, if tested undiluted, may sometimes give a false negative reaction due to the prozone phenomenon. The VDRL test can also be done as a tube flocculation test.

A simple flocuculation test, particularly useful for screening is the rapid plasma reagin (RPR) card test. This uses a carbon-containing cardiolipin antigen. A fingerprick sample of blood is sufficient. The test does not require microscopy and can be done in the field.

The major disadvantage in the standard tests is that the antigen is nonspecific and hence may react with the sera of patients who may not have syphilis. This occurrence of false positive reactions, for inherent reasons and not due to any technical error, is known as 'biological false positive' (BFP) reaction. The antibody reacting with the lipoidal antigen is known as 'reagin'. (The antibody found in atopic conditions is also called reagin, but there is no connection between the two.) Reagin, and therefore biological false positive reactions, may occur in a wide variety of infections and noninfectious conditions associated with tissue damage. These include leprosy (particularly lepromatous leprosy), malaria, relapsing fever' infectious mononucleosis, hepatitis, tropical eosinophilia and collagen diseases such as systemic lupus erythematosus and rheumatoid arthritis. Repeated blood loss, menstruation, coronary disease, severe trauma, vaccination and pregnancy may at times lead to false positive reactions. BFP reactions may be

classified as acute and chronic; in the former they disappear within six months whereas in the latter they persist indefinitely. Acute BFP reactions are seen in infections and chronic reactions in collagen diseases. It is to eliminate false positive reactions that tests have been developed using treponemal antigens.

Treponemal tests: A. Tests using Reiter treponeme: The only test in this group that is employed at present is the Reiter protein complement fixation test (RPCF). The principle is the same as the Wassermann test, but the antigen is an extract of the Reiter treponeme. This is less sensitive than the cardiolipin tests in early syphilis, but is more sensitive in late or latent syphilis. It is much more specific, but false positive reaction still occur in a small percentage of sera.

B. Tests using *T. pallidum* : (Nichol's strain): Several tests have been described. They may be classified as those in which the antigen used is live, killed or an extract of the spirochaete.

1. Tests using live *T. pallidum:* The Treponema pallidum Immobilisation (TPI) test employs live *T. pallidum* maintained anaerobically in a complex medium. The test serum is incubated anaeorbically with a suspension of the treponemes and complement. If antibodies are present, the treponemes will be found to be 'immobilised' (rendered nonmotile), when examined under dark ground illumination. The test is considered reactive if more than 50 per cent of the treponemes are immobilised, doubtful if between 50 per cent and 20 per cent are immobilised and nonreactive if less than 20 per cent are immobilised. Information about penicillin treatment should invariably be furnished as traces of penicillin will interfere with the test. TPI test is the most specific test available for syphilis However, the test is reactive in other treponemal infections like yaws.

2. Tests using killed *T. pallidum:* a Treponema pallidum agglutination (TPA) test: This employs a suspension of Nichol's strain inactivated by formalin When the serum and the antigen are incubated and examined under dark ground illumination, the treponemes are found agglutinated in the presence of antibodies. The test is not technically difficult, but is not very specific and false positive reactions are common.

b. *Treponema pallidum* immune adherence (TPIA) test: This utilises the immune adherence (von Rieckenberg) phenomenon that is used for the demonstration of antibodies in certain parasitic infection. If a suspension of treponemes is mixed with the test serum, complement and fresh heparinised whole blood from a normal individual and incubated, the treponemes will be found to adhere to the erythrocytes in the presence of antibodies. They will then be phagocytosed by leucocytes and will disappear. In the absence of antibodies, immune adherence will not occur. This test has not been widely employed as originally described, but a simplified from has been introduced recently.

c. Fluorescent treponemal antibody (FTA) test This is an indirect immunofluorescence test using as antigen, smears prepared with Nichol's strain of *T. pallidum.* Slides with smears can be stored in the deep freeze for several months. The patient's serum (diluted appropriately) is allowed to react with the smear. The excess serum is then washed off and the smear treated with antihuman immunoglobulin fluorescent conjugate. After incubation and washing off the unfixed conjugate, the slide is examined under the ultraviolet microscope. The treponemes will be seen as fluorescent objects if the test is reactive.

As originally performed, the serum was used in a dilution of 1 in 5 and at this dilution there were false positive reactions. This was therefore modified by using the serum at a dilution of 1 in 200. In this test, called FTA 200, false positive reactions were largely, but not completely, eliminated. A further modification of the test is the FTA-ABS test. Here the serum is first absorbed with an extract of Reiter treponemes (sorbent), which removes reagin and the group reactive antibody. The FTA-ABS test has been found to be almost as specific as the more complicated TPI test. Another modification, the IgM FTA-ABS test selectively identifies IgM antibodies. This helps

STS

to diagnose congenital syphilis and distinguish it from seropositivity due to passively transferred maternal antibodies (syphilotoxaemia).

3. Test using *T. pallidum* extract: The *Treponema pallidum* haemagglutination (TPHA) test developed by Rathlev (1967) employs as antigen tanned erythrocytes sensitised with an extract of *T. pallidum* obtained by freeze thawing. Cross reacting antibodies are absorbed from the sera before testing. A microtechnique (MHA-TP) suitable for automation is widely used. This test combines a high degree of sensitivity and specificity.

In some cases of neurosyphilis, reagin antibodies may be absent in serum, but may be demonstrable in the CSF. This is due to local production of antibodies in the nervous system. When a diagnosis of neurosyphilis is suspected, it is therefore important to examine the CSF for antibodies.

TABLE 42.2

Frequency of reactive serological tests in untreated syphilis (percentage)

Stage	VDRL	FTA-ABS	TPHA
Primary	70	85	55
Secondary	100	100	100
Latent or late	70	98	98

Interpretation of serological tests for syphilis Reagin antibodies appear in the serum only about four weeks after infection, so that they may not sometimes be demonstrable in the primary stage of the disease. Tests for reagin are reactive only in 50–70 per cent of patients with primary chancre. Thereafter, antibodies increase in titre, reaching maximum levels in the secondary stage, when all standard tests for syphilis are invariably reactive in high titre. During the next several years, the titre declines gradually and in the late stages of syphilis, tests for reagin may become nonreactive. The evolution of the group treponemal antibody detected by RPCF, generally parallels reagin. FTA-ABS test becomes reactive earlier than reagin tests. The specific antibody, detected by TPI test appears later so that the test is usually nonreactive in primary syphilis, and sometimes even in early secondary syphilis. But thereafter, the antibodies remain, perhaps for the rest of the patients' lifetime (Table 42.2).

The results of serological tests are influenced by specific treatment. If adequate treatment is given immediately after the primary lesion appears, the patient may never develop demonstrable antibodies and all serological tests will be nonreactive. If treatment is given in the secondary stage, reagin tests which would have become reactive by then, become nonreactive within 2–6 months. If treatment is delayed, serological tests take longer to become nonreactive, and in late syphilis, they may continue to be reactive even after complete treatment. The TPI test may not become negative after treatment, except in early stages. Hence, for the assessment of cure following treatment, reagin tests are more useful than the TPI test.

In the serological diagnosis of congenital syphilis, it is necessary to distinguish between maternal antibodies passively transferred across the placenta and the antibodies produced as a result of active fetal infection. In the former, repeated tests will show a fall in titre, whereas in the latter, the titre will increase or remain stationary. Demonstration of IgM antibody in the newborn is also indicative of active infection, as IgM does not normally cross the placenta.

The standard tests for reagin antibody are employed for the routine diagnosis of syphilis. Because of its simplicity, the VDRL test is used most often. It is desirable to perform a 'battery' of tests rather than a single test, as biological false positive reactions often give aberrant results in different tests. The FTA-ABS and TPHA tests are not technically complicated, and have found wide application because of their relative specificity. The more demanding TPI test is done only in reference laboratories and is now seldom employed for diagnosis.

Attempts have been made to develop diagnos-

tic skin tests using treponemal extracts as antigens. These have not been found useful as hypersensitivity develops irregularly and late in the disease.

Epidemiology. Venereal syphilis is worldwide in distribution. During the five centuries that it has been recorded and studied, the disease has undergone much variation in its natural history and clinical features. As originally described, it was a highly virulent disease with florid cutaneous manifestations. With the discovery of the dramatic therapeutic response to penicillin, it was hoped that it may even be possible to eradicate syphilis, as the disease has no extra human reservoir. However, not only has it not been possible to eliminate the disease, but there has occurred even an increase in its incidence, due to the changing customs, habits and values in society.

Immunity: The immune mechanisms in syphilis are not well understood. A patient is said to be refractory to reinfection so long as the original infection persists (premunition or infection-immunity). He is susceptible to reinfection after his first infection has been cured during the early stages. This accounted for the so called 'pingpong syphilis' described in sailors who used to acquire the infection ashore, and get treated aboard, only to get infected again at the next port.

Patients develop humoral antibodies, IgM, IgG and IgA, following infection, at about the time the primary chancre appears. The antibodies increase in titre during the next several months. However, even high titres of humoral antibodies do not influence the natural progression of the infection. It has, therefore, been suggested that cell mediated immunity may be more relevant in the body's defence against the treponemes. During the early stage of syphilis, there occurs anergy or inhibition of cell mediated immune response against treponemal antigens, as well as to some other antigens. Cell mediated immunity to treponemal antigens reappears after the secondary stage of syphilis.

Prophylaxis: As transmission is by direct contact, it is possible to protect against syphilis by avoidance of sexual contact with an infected individual. When this is unavoidable, as in the case of sailors on shore leave, the use of physical barriers (such as condoms), antiseptics (potassium permanganate) or antibiotics may minimise the risk. The use of prophylactic penicillin carries the danger that it may suppress the primary lesion without eliminating the infection so that recognition and treatment of the disease may become more difficult. No vaccine is available.

Treatment: Chemotherapy of syphilis has been of great interest. Mercurials, iodides, bismuth and arsenicals have been replaced by penicillin. Penicillin is uniformly effective in syphilis, but it is necessary to give an adequate dose and maintain the drug level sufficiently long to establish cure. A single injection of 2.4 million units of benzathine penicillin G is adequate in early cases. For late syphilis, this amount may be repeated weekly for three weeks. In patients allergic to penicillin, erythromycin or tetracyline may be used. Chloramphenicol is not recommended. Penicillin treatment in syphilis sometimes induces a reaction, the Jarisch-Herxheimer reaction consisting of fever, malaise and exacerbation of symptoms. It is frequent, but harmless, in primary and secondary syphilis. It is rare in late syphilis, but can be dangerous in some cases of gummatous, cardiovascular or neurosyphilis. It is believed to be due to the liberation of toxic products from the massive destruction of treponemes or due to hypersensitivity.

It has been reported that *T. pallidum* may sometimes survive in the brain and other tissues of patients clinically cured after adequate treatment with penicillin. The significance of such persistent treponemes is not clear.

NONVENEREAL TREPONEMATOSES

Nonvenereal treponemal diseases occur in endemic foci in several parts of the world, in communities with poor standards of hygiene. The dis-

eases have been given different names in different regions and vary somewhat in clinical manifestations, but the treponemes responsible are virtually indistinguishable from *T. pallidum.* Infection is usually transmitted by direct body to body contact. It has been suggested that endemic treponematoses represent the ancient patterns of association between man and treponemes When civilisation and the general use of clothing limited the chances of bodily contact among persons, the treponemes may have become adapted for transmission through sexual intercourse, resulting in venereal syphilis.

Three distinct forms of endemic treponematoses are recognised — endemic syphilis, yaws and pinta.

Endemic syphilis

Syphilis, transmitted nonvenereally, was endemic in several foci. With recognition of such foci and mass treatment with penicillin under WHO auspices, endemic syphilis has become very rare. Under the name of *sibbens,* it was present in Scotland in the last century. It is known as *bejel* in the Middle East, *njovera* in Zimbabwe, *dichuchwa* in Bechuanaland, *skerljevo* in Eastern Europe and *siti* in Gambia It has also been reported from India.

The disease is common in young children Primary chancre is not usually seen, except sometimes on the nipples of mothers infected by their children. The disease is usually seen with manifestations of secondary syphilis, such as mucous patches and skin eruptions. The disease progresses to tertiary lesions, particularly gummatous lesions. Cardiovascular and neurological involvement is rare. Congenital syphilis also is not found.

The laboratory diagnosis and treatment are as for venereal syphilis.

Only secondary.

Yaws

Yaws, also known as framboesia, pian, parangi and by many other synonyms, is endemic in the tropical areas of Africa, Asia and America. Yaws eradication campaign by mass penicillin injections in endemic areas, organised in the 1980's led to the virtual eradication of the disease. But it has subsequently reappeared in some areas. In India cases have been identified in Andhra Pradesh, Orissa and Madhya Pradesh. The causative agent is *T. pertenue,* which is morphologically and antigenically indistinguishable from *T. pallidum.* The primary lesion (mother yaw) is an extragenital papule which enlarges and breaks down to form an ulcerating granuloma. As in syphilis, secondary and tertiary manifestations follow, but cardiovascular or neurological involvement is rare. Destructive gummatous lesions of the bones are common.

Infection is by direct contact. Flies may act as mechanical vectors. The small fly, *Hippolates pallipes,* has been found feeding on open sores, but its epidemiological importance is not known.

Laboratory diagnosis and treatment are as for syphilis. There appears to be some cross immunity between yaws and syphilis in that venereal syphilis is rare in communities where yaws in endemic.

Pinta

Pinta *(carate, mal del pinto)* is endemic in Central and South America and the neighbouring islands. The primary lesion is an extragenital papule, which does not ulcerate, but develops into a *lichenoid or psoriaform* patch. *Secondary skin* lesions are characterised by hyperpigmentation or hypopigmentation.

The causative agent is *T. carateum* (also called *T herrejoni).* It is very closely related to *T. pallidum,* but is not antigenically identical so that cross immunity between pinta and syphilis is only partial.

Nonpathogenic treponemes

Several commensal treponemes occur on the buccal and genital mucosa and may cause confusion in the diagnosis of syphilis by dark field examina-

tion. These include *T. microdentium* and *T. macrodentium*, found in the mouth. The former resembles *T. pallidum* closely, but is shorter (3–6μ) with shallower coils. The latter is larger, thicker and more actively motile with irregular coils.

T. refringens was observed by Schaudinn and Hoffamann when they first discovered *T. pallidum*. It was found in syphilitic as well as non-syphilitic genital lesions. It has been described as having a wavy middle part, but more regularly and deeply curved extremities, terminating in pointed ends. *T. calligyrum (T. gracile)* which also may be found in genitalia is thicker and has shallower spirals.

In experimental work on *T. pallidum* infection in rabbits, it is important to keep in mind a natural venereal infection in rabbits caused by *T. cuniculi*, which is morphologically identical with *T. pallidum*.

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43 Mycoplasma

The causative agent of bovine pleuropneumonia, first isolated by Nocard and Roux (1898), was found to be a filterable and highly pleomorphic microorganism, varying in shape from spherical or ovoid bodies to branching filaments resembling fungal mycelia. Another such organism was isolated from contagious agalactia of sheep. Subsequently, several such morphologically similar organisms were isolated from animals and man, as well as from soil and sewage. Because of their resemblance to the organism causing pleuropneumonia, they were called 'pleuropneumonia-like organisms' or PPLO. This unsatisfactory term has been replaced by the name *Mycoplasma*, which refers to the fungus-like form of the branching filaments.

The plasticity of mycoplasma is due to the absence in them of a rigid cell wall. Such microorganisms devoid of a cell wall or cell wall precursors, such as muramic acid and diaminopimelic acid, and bound by a single triple layered membrane have been placed in the class *Mollicutes* (literally meaning 'soft skin'), order *Mycoplasmatales.* Parasitic mycoplasmas, requiring cholesterol or other sterols as an essential growth factor, belong to the Family *Mycoplasmataceae.* This contains two genera, the Genus *Mycoplasma* which utilises glucose or arginine, but does

TABLE 43.1

Mycoplasmas of humans

A. Parasitic:

I. *Frequently pathogenic*
Mycoplasma pneumonia causes pneumonia, tracheo-bronchitis; associated with arthritis

II *Occasionally pathogenic*
M. hominis associated with post partum fever, salpingitis, endometritis, abortion, sterility, septicaemia, abscesses, arthritis, urethritis.
Ureaplasma urealyticum, associated with nongonococcal urethritis, pelvic inflammation, septicaemia, arthritis

III. *Nonpathogenic*
M. orale, M. salivarium, M. buccale, M. faucium found in oropharynx: *M. fermentans, M. primatum* found in genital tract.

B. Saprophytic:
Acholeplasma laidlawii, may be found on skin and in mouth.

not hydrolyse urea, and the Genus *Ureaplasma* which hydrolyses urea. Saprophytic mycoplasmas found in sewage and soil do not require sterols for growth and are placed in the Family *Acholeplasmataceae*, Genus *Acholeplasma*. A third Family *Spiroplasmataceae* contains helical shaped spiroplasmas which are arthropod borne pathogens of plants. Mycoplasmas may be saprophytic, parasitic or pathogenic. More than 60 species of mycoplasma are known to cause disease in a variety of mammalian, insect and plant hosts. Some ten species belonging to three genera may be found in humans (Table 43.1).

Morphology: Mycoplasmas are the smallest free living microorganisms, and one of the most pleomorphic (Fig. 43.1). They occur as granules and filaments of various sizes. The granules may be minute 'elementary bodies' about 125–250 nm in diameter or larger forms about 500–1000 nm in size, with balloon, disc, ring or star shape. The filaments are slender, of varying lengths and show true branching. Some strains are coccobacillary. The method of reproduction is not fully understood, but appears to be 1) by the development within the filaments of elementary bodies and their subsequent release by fragmentation and disintegration of the filaments, 2) by binary fission, and 3) by budding. No spores or flagella are seen. They are generally nonmotile, though a gliding motility has been described in some species.

The characteristic feature is the complete absence of cell wall and its components. Due to their plastic form and the small size of the minimum reproductive particle, they are filterable and therefore have often been mistaken for viruses. The mycoplasma cell is surrounded by a single triple-layered membrane which is rich in cholesterol and other lipids. Some species have a 'foot-like' appendage on the membrane by means of which they get attached to suitable animal cells carrying neuraminic acid receptors. They are also believed to be responsible for the haemadsorption shown by some species of mycoplasma.

Mycoplasmas are Gram negative, but are better stained by Giemsa stain.

Cultivation: Mycoplasmas may be cultivated in fluid or solid media. They are generally facultative anaerobes, growth being better aerobically. They grow within a temperature range of 22°–41°C, the parasitic species growing optimally at 35°–37°C and the saprophytes at lower temperature. Media for cultivating mycoplasma are enriched with 20 per cent horse or human serum and yeast extract. Penicillin and thallium acetate are added as selective agents. The high concent-

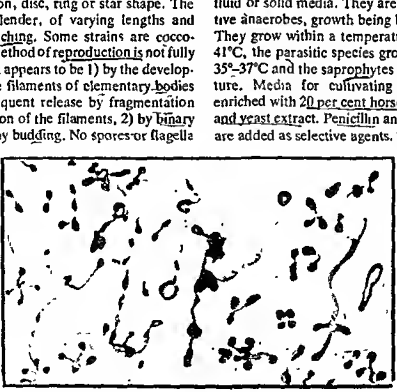

Fig. 43.1 Morphology of Mycoplasma—extreme pleomorphisms with branched, filamentous, beaded and swollen forms

heart infusion peptone broth c̄ 2% agar

ration of serum is necessary as a source of cholesterol and other lipids. Colonies appear after incubation for 2–3 days and are on an average about 10–600 μ in size. The colony is typically biphasic, with a 'fried egg' appearance, consisting of a central opaque granular area of growth extending into the depth of the medium, surrounded by a flat, translucent peripheral zone (Fig. 43.2) Colonies may be seen with a hand lens, but are best studied after staining by Dienes method. For this, a block of agar containing the colony is cut and placed on a slide. It is covered with a cover slip on which has been dried an alcoholic solution of methylene blue and azure.

Colonies cannot be picked with platinum loops. Subculture is done by cutting out an agar block with colonies and rubbing it on fresh plates Most mycoplasma colonies are haemolytic.

Biochemical reactions: Mycoplasmas are chemoorganotrophs, the metabolism being mainly fermentative. Most species utilise glucose or arginine as the major sources of energy. Urea is not hydrolysed, except by ureaplasmas They are generally not proteolytic.

Resistance: Mycoplasmas generally resemble nonsporing bacteria in heat resistance, but some strains are more sensitive, being destroyed at 45°C in 15 minutes They are relatively resistant to lysis by osmotic shock but are very sensitive to lysis by surface active agents and lipolytic agents such as taurocholate and digitonin. They are resistant to penicillin and cephalosporin as well as to lysozyme that act on bacterial cell walls, but are sensitive to tetracycline and many other antibiotics. Susceptibility to erythromycin and some other macrolide antibiotics is useful for species differentiation. Growth is inhibited by organic gold salts. *M. pneumoniae* can grow in the presence of 0.002 per cent methylene blue in agar, while many other species are inhibited.

Antigenic properties: Serological tests such as complement fixation, agglutination, passive haemagglutination and immunofluorescence have been employed for detection of antibodies in sera and for identification of isolates. A particularly useful technique for identification of isolates is the growth inhibition test based on the ability of antisera to specifically inhibit the growth of the homologous species on solid media.

Mycoplasmas and L forms of bacteria: Kleineberger (1935) found pleuropneumonia-like forms in a culture of *Streptobacillus moniliformis* and termed them L forms, after Lister Institute, London, where the observation was made It was subsequently shown that many bacteria, either spontaneously or induced by certain substances like *penicillin*, lost part or all of their cell wall and develop into L forms Such L forms may be 'unstable', when they revert to their normal morphology, or 'stable', when they continue in the cell wall deficient state permanently. Cell wall deficient forms (L forms, protoplasts, spheroplasts) may not initiate disease, but may be important in bacterial persistance during antibiotic therapy and subsequent recurrence of the infection

It has been suggested that mycoplasmas may represent stable L forms of bacteria. The latter may also produce 'fried egg' colonies-like mycoplasma, but they differ in a number of respects. L forms resemble the parent bacteria biochemically and antigenically; they are not filterable, the

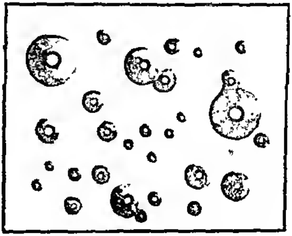

Fig. 43 2 'Fried egg' appearance of mycoplasma colonies

minimum reproductive unit being about 600 nm; though L forms lack cell walls, the cell wall components may be demonstrated. Sterols are not required for the growth of L forms; they are nonpathogenic and they show nucleic acid homology and have the same base ratios as the parent bacteria.

As agglutinins to *Streptococcus* MG are frequently developed following infection with *M. pneumoniae*, it has been suggested that the latter is an L form of the former, but all available evidence is against this hypothesis.

Ureaplasma urealyticum. Some strains of mycoplasma frequently isolated from the urogenital tract of man and animals form very tiny colonies, generally 15–25μ in size. They were called T strain or T form mycoplasmas (T for tiny). They are peculiar in their ability to hydrolyse urea, which is an essential growth factor in addition to cholesterol. Human T strain mycoplasmas are now classified as *Ureaplasma urealyticum*. They are highly susceptible to erythromycin and are more susceptible to thallium acetate than other mycoplasmas. They have been proposed as the causative agents in nongonococcal urethiritis and Reiter's syndrome, but their aetiological role is not proven.

Pathogenicity: Mycoplasma can cause two diseases in man — pneumonia and genital infections. Mycoplasmal pneumonia (primary atypical pneumonia) is caused by *M. pneumoniae*. The disease has an incubation period of 1–3 weeks. The onset is gradual, with fever, malaise, headache and sore throat. Paroxysmal cough may occur with blood tinged sputum. The disease is characterised by paucity of respiratory signs on physical examination, but marked radiological evidence of consolidation, which is usually unilateral, involving the lower lobe, and starting at the hilum, and fanning out to the periphery. The disease is self-limited, recovery occurring in 1-2 weeks. Bullous myringitis and otitis are common complications. Rashes, meningitis, encephalitis and haemolytic anaemia are other complications seen.

The disease is worldwide and is found commonly in older children and adolescents. It is common among military recruits. Transmission is by droplets of nasopharyngeal secretions. The mycoplasma may remain in the throat for two or more months after recovery from the disease.

Eaton (1944) was the first to isolate the causative agent of the disease in hamsters and cotton rats. He was able to transmit the infection later to chick embryos by amniotic inoculation. Because it was filterable, it was considered to be a virus (Eaton agent). It was subsequently shown to be a mycoplasma and was named *M. pneumoniae*.

Laboratory diagnosis of primary atypical pneumonia may be established either by isolation of the mycoplasma or by serological methods. For isolation, throat swabs or respiratory secretions are inoculated into the mycoplasma medium containing glucose and phenol red. Growth is slow on primary isolation and may take 5–10 days. Growth is indicated by acid production in the medium. *M. pneumoniae* produces beta haemolysis and agglutinates guinea pig erythrocytes. Colonies on agar adsorb erythrocytes. The haemadsorption is enzymatic and occurs optimally at 37°C. The cell receptors are destroyed by neuraminidase. It inhibits ciliary motility in hamster trachea organ cultures. *M. pneumoniae* is unrelated to other human mycoplasmas and may be identified by growth inhibition by specific antisera.

Serological diagnosis may be made by specific tests using mycoplasmal antigens or by nonspecific methods. Among the former, immunofluorescence, haemagglutination inhibition and metabolic inhibition are the most sensitive tests. Complement fixation and indirect haemagglutination tests are less sensitive.

The nonspecific serological tests are *Streptococcus* MG and cold agglutination tests. The former is done by mixing serial dilutions of unheated patient's serum and a heat killed suspension of *Streptococcus* MG, and observing agglutination after overnight incubation at 37°C. A titre of 1:20 or over is considered suggestive.

The cold agglutination test is based on the appearance in a high proportion of cases with

primary atypical pneumonia, of macroglobulin antibodies that agglutinate human group O cells at low temperature. The patient's blood sample should not be refrigerated before separation of the serum, as the agglutinins are readily absorbed by the homologous erythrocytes at low temperatures. For the test, serial dilutions of the patient's serum are mixed with an equal volume of a 0.2% washed human O group erythrocytes, and clumping observed after leaving at 4°C overnight. The clumping is dissociated at 37°C. A titre of 1:32 or over is suggestive, but demonstration of rise in titre in paired serum samples is more reliable. The indirect Coombs test may also be positive in some cases.

Genital infections are caused by *M. hominis* and *U. urealyticum*. They are transmitted by sexual contact. They may cause urethritis, proctitis, balanoposthitis and Reiter's syndrome in males, and acute salpingitis, pelvic inflammatory disease, cervicitis and vaginitis in females. They have also been associated with infertility, abortion, postpartum fever, chorioamnionitis and low birthweight of infants.

Mycoplasma as cell culture contaminants: Continuous cell cultures maintained in many laboratories have been found to be contaminated with different species of mycoplasma. The contamination may originate from the worker or from animal sera or trypsin used in cell culture. Contamination generally does not produce cytopathic effects but may interfere with the growth of viruses in such cell cultures and may also produce misleading results in serological tests. Mycoplasmas growing in cell cultures have often been mistaken for viruses. Eradication of mycoplasmas from infected cells is difficult.

Treatment: Tetracycline and erythromycin are the drugs of choice for treatment of mycoplasmal infections.

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Age incidence
< 5-20 yr

Majority of non gonococcal urethritis are caused by Chlamydia trachomatis

M. pneumoniae - principal cause of non bacterial pneumonia.

44 Actinomycetes

Actinomycetes are traditionally considered to be transitional forms between bacteria and fungi. Like fungi, they form a mycelial network of branching filaments, but like bacteria, they are thin, possess cell walls containing muramic acid, have prokaryotic nuclei and are susceptible to antibacterial antibiotics. They are, therefore, true bacteria, bearing a superficial resemblance to fungi. Actinomycetes are related to mycobacteria and corynebacteria. They are Gram positive, nonmotile, nonsporing, noncapsulated filaments that break up into bacillary and coccoid elements. Most are free living, particularly in the soil.

The classification of actinomycetes is not finally settled. Actinomycetes contain two medically important genera, *Actinomyces* and *Nocardia*. The genus *Streptomyces* also contains a few pathogenic species, but its medical importance lies in the production of antibiotics by several species. *Actinomyces* are anaerobic or microaerophilic and nonacid fast. *Nocardia* are aerobic and may be acid fast.

Actinomyces

Bollinger (1877) found a mould-like organism in the lesions of 'lumpy jaw' (Actinomycosis) in cattle. The name actinomyces was coined by Harz to refer to the ray-like appearance of the organism in the granules that characterise the lesions (*actinomyces*, meaning ray fungus). Wolff and Israel (1891) isolated the organism from pathological material under anaerobic conditions. Bostroem (1891) isolated from similar lesions an aerobic actinomycete, which was known as *A. hominis* (*A. graminis*).

Actinomycosis in cattle is caused mainly by *A. bovis* and in man by *A. israelii*. *A. eriksonii* has been isolated from pulmonary actinomycosis. *A. naeslundii* is normally a mouth commensal, but may occasionally cause human infection.

Actinomycosis

The disease is a chronic granulomatous infection occurring in man and animals. It is characterised by the development of indurated swellings, mainly in the connective tissue, suppuration and the discharge of 'sulphur granules'. The lesion often points towards the skin, leading to multiple sinuses. Actinomycosis in man occurs in three main clinical forms: 1) cervicofacial, with indurated lesions on the cheek and submaxillary regions; 2) thoracic, with lesions in the lung that may involve the pleura and pericardium and spread outwards through the chest wall, and 3) abdominal, where the lesion is usually around the caecum, with involvement of the neighbouring tissues and the abdominal wall. Sometimes the infection spreads to the liver via the portal vein.

Many cases of pelvic actinomycosis have been reported in association with the use of intrauterine devices. Actinomyces have been incriminated in inflammatory diseases of the gums (gingivitis and periodontitis) and with sublingual plaques leading to root surface caries. Actinomycosis may also present as mycetoma.

Laboratory diagnosis: The diagnosis is made by

demonstrating actinomycetes in the lesion by microscopy and by isolating it in culture. The specimen to be collected is pus. In pulmonary disease, the sputum is collected. 'Sulphur granules' may be demonstrated in pus by shaking it up in a test tube with some saline. On standing, the granules sediment and may be withdrawn with a capillary pipette. Granules may also be obtained by applying gauze pads over discharging sinuses.

The granules are white or yellowish and range in size from minute specks to about 5 mm. They are examined microscopically under a coverslip. They are crushed between slides and stained by Gram stain and examined. The granules are, in fact, bacterial colonies and will be found to consist of a dense network of thin Gram positive filaments, surrounded by a peripheral zone of swollen radiating club shaped structures, presenting a sun-ray appearance. The 'clubs' are suggested to be antigen-antibody complexes.

Sulphur granules or pus containing 'Actinomycetes' are washed and inoculated into thioglycollate liquid medium or streaked on brain heart infusion agar and incubated anaerobically at 37°C. In thioglycollate *A. bovis* produces general turbidity whereas *A. israelii* grows as fluffy balls at the bottom of the tube. On solid media

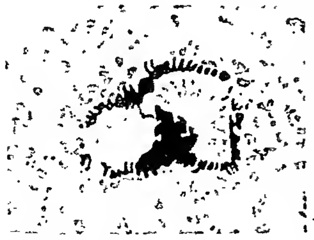

Fig 44.1 Sulphur granule. Section of tissue showing an actinomycotic colony, the club at the periphery giving a 'Sun ray' appearance

A. israelii produces small 'spidery colonies' in 48–72 hours that becomes heaped up, white and irregular, or smoother, larger colonies in 10 days. Other species have different types of colonies.

The isolate is identified by microscopy, biochemical reactions and fluorescent antibody methods. Gel diffusion and immunofluorescence can differentiate *A. israelii* from other actinomycete species and from other filamentous anaerobes that may produce granules in tissues.

Epidemiology: Actinomycosis in man is an endogenous infection. The bacillus is normally present in the mouth as a commensal. Trauma or poor oral hygiene may predispose to tissue invasion.

The disease occurs throughout the world, but its incidence in the advanced countries has been declining in the last few decades, probably as a result of the widespread use of antibiotics. Actinomycosis is more common in rural areas and in agricultural workers. Young males (10–30 years old) are most commonly affected. The reason for this predisposition is not known. About 60 per cent of cases are cervicofacial and some 20 per cent abdominal.

In actinomycotic lesions, a small Gram negative aerobic bacillus, *Actinobacillus actinomycetemcomitans* is usually found, sometimes in very large numbers. It may have a symbiotic role in pathogenesis.

Treatment. Most cases respond to medical treatment with streptomycin, cotrimoxazole, pyrimethamine and rifampicin. Treatment may have to be prolonged for several months. Surgery may become necessary if medical treatment fails.

Nocardia

Nocardia resemble *Actinomyces* morphologically but are aerobic. All species are Gram positive and some, *N. asteroides* and *N. brasiliensis*, also acid fast. These may cause respiratory disease and appear in the sputum, where they may be mistaken for tubercle bacilli. Smears show

branching filaments which may fragment to form bacillary or coccoid elements.

Nocardia species are frequently found in soil and infection may be exogenous. Nocardiosis may begin as a pulmonary infection that may be subclinical or produce pneumonia. The localised lesion may remain chronic as an enlarging abscess, sinus tract or cavity. There is a tendency to brain abscesses as a result of haematogenous spread. Lesions may develop in the kidney. Immunodeficiency makes persons prone to nocardiosis. *N. madurae* which is nonacid fast causes mycetoma.

Diagnosis is by demonstration of branching filaments microscopically and by isolation in culture. Nocardia grow readily on ordinary media forming dry, granular, wrinkled colonies which produce pigment ranging from yellow to red.

Nocardia are sensitive to sulphonamides and nalidixic acid which have been used in treatment. Chemotherapy has not in general been very successful.

Actinomycotic mycetoma

Mycetoma is a localised, chronic, granulomatous involvement of the subcutaneous and deeper tissues, affecting commonly the foot and less often the hand and other parts, and presenting as a tumour with multiple discharging sinuses. This clinical syndrome was first described from Madura by Gill (1842) and came to be known as Maduramycosis. Mycetomas are usually caused by fungi, but may be caused by bacteria as well. Even *Staph. aureus* and other pyogenic bacteria may occasionally cause a mycetoma-like lesion *(botryomycosis)*. Bacterial mycetomas are usually caused by actinomycetes — *Actinomyces (A. israelii, A. bovis), Nocardia (N. asteroides, N. brasiliensis, N. caviae, N. madurae), Streptomyces (S. pelletierii, S. somaliensis)*.

Aetiological diagnosis of mycetomas is important in choosing appropriate treatment. The colour of the granules gives some indication. In actinomycotic mycetoma, the granules are white to yellow, while in mycotic mycetomas, the granules are generally black. Examination of crushed smears of the granules helps to differentiate actinomycotic from mycotic mycetomas. In the former the filaments are thin (about 1μ), while in the latter they are stout (about $4-5\mu$). Isolation of the agent in culture establishes the diagnosis.

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45 Miscellaneous Bacteria

monocytosis ✓
Anton test
Infn from Animal Cont
Ampicillin
GB at 4°c, Sub/c wkly 1-6 weeks

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a small, coccoid, Gram positive bacillus, with a tendency to occur in chains. Rough forms may be seen as long filaments. It exhibits a characteristic, slow, tumbling motility when grown at 25°C, but at 37°C is non-motile. This is because peritrichous flagella are produced by the bacillus optimally at 20°C–30°C, but only scantily or not at all at 37°C. It is aerobic or microaerophilic. Growth is improved when cultures are incubated at reduced oxygen tension and with 5–10 per cent CO_2. It grows on ordinary media within a temperature range of 4°C – 42°C. Growth is improved by the addition of glucose, blood or liver extract. Colonies are haemolytic on blood agar. It ferments glucose and salicin promptly producing acid without gas; lactose, maltose and sucrose are fermented slowly or not at all, and mannitol is not fermented. It is catalase positive. It is unusually resistant to heat, being killed only in an hour at 55°C. It grows in the presence of 0.1% potassium tellurite, 10% salt and at pH 9.6. At least four main serotypes are distinguishable by H and O antigens.

L. monocytogenes is widely distributed in nature. It has been isolated from a wide range of mammals, birds, fish, ticks and crustacea. It occurs as a saprophyte in soil, water and sewage. Listeriosis in humans may present in many forms. It may cause meningitis or meningoencephalitis, particularly in neonates and in persons over 40 years. Infection of pregnant women may lead to abortion or stillbirth. Asymptomatic infection of the female genital tract may cause infertility. Listeriosis may also present as abscesses, conjunctivitis, pharyngitis, urethritis, pneumonia, infectious mononucleosis-like syndrome, endocarditis or septicaemia.

Most human infections are caused by serotypes 1b and 4b. Experimental inoculation in rabbits causes a marked monocytosis (hence the name *monocytogenes*). Monocytosis is a feature of human listeriosis also. Instillation into the eyes of rabbits produces a keratoconjunctivitis (Anton test). Human infection is believed to result from contact with infected animals, inhalation of contaminated dust or ingestion of contaminated milk.

Laboratory diagnosis is established by the isolation of the bacillus from appropriate clinical material such as cervical and vaginal secretions, lochia, meconium, cord blood, blood and cerebrospinal fluid. Greater success in isolation is achieved if the materials are stored in glucose broth at 4°C and subcultures are done at weekly intervals for 1–6 months. Listeriosis in man is being increasingly reported. Isolates are likely to be missed as nonpathogenic diphtheroids unless properly investigated. Ampicillin is said to be the most effective antibiotic both *in vitro* and *in vivo*. Penicillin, erythromycin, tetracycline and sulphadiazine all inhibit the growth of the organism.

ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix rhusiopathiae is a slender, nonmotile, nonsporing, noncapsulated Gram positive rod, with a tendency towards formation of

long filaments. It is microaerophilic on primary isolation, but on subculture, grows as an aerobe or facultative anaerobe. It grows on ordinary media. Growth is improved by addition of glucose. Heart infusion agar with rabbit blood and 5% CO_2 is good for primary isolation. Black colonies are developed in tellurite media. It ferments glucose and lactose, producing acid without gas; sucrose and mannitol are not fermented. Different antigenic types have been recognised.

E. rhusiopathiae is a natural parasite of many animals. It causes swine erysipelas and human erysipeloid. Human Infection usually occurs on the hand or fingers of persons handling animals, fish or animal products. The lesions are painful, oedematous and erythematous, usually involving the local lymph glands and joints. Occasional cases of endocarditis have been reported. The bacillus is sensitive to penicillin, erythromycin and broad spectrum antibiotics.

ALKALIGENES FAECALIS

The name *Bacterium faecalis alkaligenes* was originally applied to an ill defined group of Gram negative bacilli isolated from human faeces, which did not ferment sugars, but produced an alkaline reaction in litmus milk. The term *Alkaligenes faecalis* now refers to Gram negative, short, nonsporing bacilli, which are strict aerobes and do not ferment sugars. They are motile by means of peritrichous flagella. They are usually oxidase positive. Nitrate reduction is variable.

Alk. faecalis is a saprophyte found in water and soil contaminated with decaying organic matter. They are also commensals in the intestines of man and animals. They have been isolated from a variety of clinical specimens such as urine, pus and blood. They have been considered responsible for a typhoid-like fever, urinary infections, infantile gastroenteritis and suppuration in various parts of the body.

CHROMOBACTERIUM VIOLACEUM

Chromobacterium violaceum is a Gram negative, nonsporing bacillus, motile by means of polar and lateral flagella. They are facultative anaerobes growing on ordinary media and producing a violet pigment soluble in ethanol and insoluble in water and chloroform. They are oxidase negative. They are saprophytic in water and soil. Human infections have been recorded mainly in the tropics and consist of skin lesions with pyaemia and multiple abscesses.

FLAVOBACTERIUM MENINGOSEPTICUM

Flavobacterium meningosepticum is a Gram negative nonmotile rod, producing a yellowish pigment. It is a ubiquitous saprophyte capable of causing opportunistic infections. It has been responsible for outbreaks of meningitis in newborn infants. Infection in adults leads to a mild febrile illness.

DONOVANIA GRANULOMATIS
(Calymmatobacterium granulomatis)

Donovan (1905) described the presence of characteristic intracellular bodies in smears from ulcerated lesions of a disease now known as Donovanosis. He considered the bodies to be parasites. Donovanosis is a venereal disease seen mainly in the tropics, in which slowly progressive ulceration of genitalia occurs. Donovan's intracellular bodies have since been identified as bacteria and have been named *Donovania granulomatis*.

D. granulomatis is a pleomorphic rod with rounded ends and bipolar condensation of chromatin giving rise to a 'safety pin' appearance. It is usually capsulated and can be stained by Wright's stain, appearing as blue bacillary bodies surrounded by well defined pink capsules. It is nonmotile and Gram negative. It can be grown on egg yolk medium and on a modified Levinthal agar. It is morphologically and antigenically similar to klebsiellae.

Pathogenicity is limited to man. The disease develops after a variable incubation period ranging from a few days to three months. Intradermal

inoculation of whole cultures or of an alkaline extract of cultures in patients produces a red oedematous reaction in 24 hours. Streptomycin and tetracyclines are useful in treatment.

ACINETOBACTER
(Mima polymorpha; Bacterium anitratum)

De Bord (1939) proposed the name Mimeae for a new tribe of bacteria made up of short Gram negative encapsulated pleomorphic rods. The name 'Mimeae' referred to their mimicking neisseriae in appearing as Gram negative diplococci and causing urethritis and conjunctivitis. Two members of the tribe Mimeae — *Mima polymorpha* and *Bacterium antitratum* have been frequently isolated from a large variety of clinical conditions. They have been responsible for meningitis, septicaemia and urinary, genital, respiratory and wound infections.

These bacilli are aerobes and grow well on ordinary media. They do not reduce nitrates. A variety of names have been employed by different investigators for these bacteria. According to the current classification, they are grouped under the genus *Acinetobacter*, which comprises two distinct species, *Ac. anitratus* (corresponding to *Bact. anitratum*) and *Ac. lwoffi* (corresponding to *M. polymorpha*).

Ac. anitratus: These form pinkish colonies on MacConkey's medium. Acid without gas is formed in *glucose, arabinose, xylose,* and occasionally in rhamnose. A characteristic reaction is the formation of acid in 10%, but not 1% lactose. Several serotypes have been identified by capsule swelling and immunofluorescence.

Ac. lwoffi: This forms yellow colonies on MacConkey's medium and does not acidify sugars. Some strains are oxidase positive.

Acinetobacter species are opportunist pathogens and are often found in hospital infections, particularly in iatrogenic meningitis. They are frequently present on the normal skin. All strains are resistant to penicillin, but most strains are sensitive to one or the other of the broad spectrum antibiotics.

STREPTOBACILLUS MONILIFORMIS

Streptobacillus moniliformis is a microaerophilic, pleomorphic, Gram negative bacillus that grows on artifical media as tangled chains of rods or various lengths, with beaded or fusiform swellings. It develops readily into L forms. In fact, it was during the study of this bacillus that L forms were originally discovered.

Str moniliformis is a natural parasite of rodents and produces one form of rat bite fever in man (the other variety being caused by *Spirillum minus*). The disease may be caused either by rat bite or by drinking milk contaminated with rat excreta. The latter variety of infection can occur as outbreaks and is known as Haverhill fever. Clinically, it is a persistent type of fever with polyarthritis and a petechial or morbiliform rash. The bacillus can be grown on media containing blood, serum or ascitic fluid, aerobically under increased CO_2 tension.

SPIRILLACEAE

The family spirillaceae consists of two genera *Spirillum* and *Campylobacter*, both of which contain organisms pathogenic to man. *Spirillum minus is one of the causes of rat bite fever* and is the sole human pathogen of the genus. *Campylobacter species* may cause neonatal septicaemia, severe diarrhoea, and a variety of other infections. The importance of this organism has only been recently recognised.

Spirillaceae are rigid helically curved rods with a variable number of spirals. They are motile by means of flagella and move in a corkscrew fashion. Most of the organisms in the family are free living in fresh or salt water. Other are saprophytic or parasitic and human or animal pathogens. *Spirillum* species have flagella at both poles and are strict aerobes.

Spirillum minus: It is a short, thick organism with tapering ends. It is 0.2–0.5 μ x 3–5 μ in size with regular spirals, usually two to three. It is motile by amphitrichous flagella. It is Gram negative but is better visualised by Wright's or Giemsa or Fontana stain and by dark ground microscopy.

It is a natural parasite of rats and other rodents. It causes one type of rat bite fever known as *Sodoku*. After an incubation period of about two weeks, the fever sets in abruptly, accompanied by an erythematous rash at the site of the rat bite. The rash at the site may progress to a chancre like lesion. Regional lymph glands are usually enlarged. Mortality rate of 5–10 per cent has been reported.

The organism has not been cultivated *in vitro*. Diagnosis may be made by the microscopic examination of the exudates from lesions or by the inoculation of the exudates, lymph node aspirates or blood into white mice or guinea pigs. It should be ensured that the mice do not already harbour any spirilla. Penicillin and streptomycin are useful in treatment.

CAMPYLOBACTER

The genus *Campylobacter* contains slender spirally curved Gram-negative rods, 0.2–0.5μ thick and 0.5–5μ long. They are typically comma shaped, but may occur as S- or multispiral chains. Old cultures are coccoid and pleomorphic. They are nonsporing and motile with a single polar flagellum at one or both poles. Growth occurs under micro-aerophilic conditions, 5% O_2 concentration being optimal. Many pathogenic species are thermophilic, growing well at 42°C. Campylobacters do not attack carbohydrates, but are oxidase positive.

Campylobacters have gained prominence recently as a common cause of human diarrhoeal disease, affecting children and adults. They can, on occasion, also cause systemic infections. They are important veterinary pathogens. Campylobacters of medical importance are the following:

1. Causing diarrhoeal disease: *C. jejuni, C. coli, C. laridis*.
2. Causing extra-intestinal infection: *C. fetus*
3. Isolated from mouth: *C. sputorum, C. concisus*
4. Associated with gastritis and peptic ulcer ('Pyloric campylobacters'): *C. pylori*

Campylobacter jejuni

Medically, this is the most important campylobacter species as it causes innumerable attacks of diarrhoea worldwide. The infection is zoonotic, the source being food of animal origin, especially raw milk. It appears to be part of the normal intestinal flora of domestic animals and birds, and is shed in their faeces. It can be isolated frequently from surface waters.

Infection occurs by ingestion. *C. jejuni* can produce diarrhoea by three different mechanisms—production of a toxin resembling cholera enterotoxin causing watery diarrhoea; shigella-like penetration of gut epithelium leading to bloody diarrhoea; and salmonella-like penetration of mucosa with proliferation in lamina propria and mesenteric lymph nodes. The common clinical manifestations are abdominal pain, fever and profuse, watery foul smelling diarrhoea, sometimes with blood and mucus. Vomiting is not common. The disease is self-limited. Erythromycin has been reported useful in treatment, but resistant strains may occur.

Laboratory diagnosis depends on the isolation of the campylobacter from faeces. Direct microscopic examination—phase contrast or darkfield microscopy to detect the darting or tumbling motility of the spiral rods, or demonstration of the small curved rods in stained smears—may be useful for presumptive rapid diagnosis. Faeces or rectal swabs are plated on selective media. In case of delay in culturing, a transport medium has to be employed. Campylobacters survive for 1–2 weeks at 4°C in Cary-Blair transport medium, but glycerol-saline is not satisfactory. The plating media commonly used are Skirrow's, Butzler's or Campy BAP selective media. *C. jejuni*, as well as *C. coli* and *C. laridis*, are thermophilic and do not grow at 25°C. Inoculated plates are incubated at

42°C in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Thermophilic campylobacters can grow well at 37°C also, but incubation at the higher temperature suppresses normal faecal flora to some extent.

Colonies appear usually by 48 hours. They are nonhaemolytic, grey or colourless, moist, and flat or convex. Suggestive colonies are screened by Gram staining, motility and oxidase tests. Confirmation is by further biochemical tests, including positive catalase and nitrate reduction tests.

C. coli causes an infection clinically indistinguishable from that due to *C. jejuni*. *C. coli* is believed to account for 3–5 per cent of campylobacter diarrhoeas. *C. coli* is commonly found in healthy pigs. It is differentiated from *C. jejuni* by the hippurate hydrolysis test which is negative in case of *C. coli*.

C. laridis also causes a similar diarrhoeal disease, though its prevalence has not been worked out. It can be distinguished from *C. jejuni* and *C. coli* by its resistance to nalidixic acid.

C. jejuni and *C. coli* can be serotyped for epidemiological purposes.

C. jejuni is the most common bacterial cause of diarrhoeal disease in many developed countries — more common than salmonellae or shigellae. But in the developing countries, clinical disease caused by *C. jejuni* is much less frequent, though asymptomatic infection is common in humans as well as domestic animals and birds.

Some nonthermophilic campylobacters, provisionally designated *C. cinaedi* and *C. fennelliae* have been associated with diarrhoeal disease in homosexual men, but their clinical significance remains to be established.

Campylobacter fetus

This organism was isolated in 1918 by Theobald Smith from infectious abortion in cattle and named *Vibrio fetus*. It is a very important veterinary pathogen. Human infection by *V. fetus* was first observed in 1947 and subsequently also by similar organisms which came to be called 'related vibrios'. The heterogenous group was renamed *Campylobacter fetus* and classified into three subspecies.

C. fetus subsp. *jejuni* to which the 'related vibrios' belonged has been separated as the species *C. jejuni*, described above.

C. fetus subsp. *fetus* (formerly called subsp. *intestinalis*) can sometimes produce extra-intestinal disease in humans, particularly septicaemia in previously ill subjects. It causes abortion in cattle and sheep.

C. fetus subsp. *veneralis* does not appear to cause illness in man, but is responsible for infectious abortion transmitted venereally in cattle.

C. fetus is not thermophilic and grows at 25°C also.

C. sputorum and *C. concisus* have been isolated from the human oral cavity, but not associated with any disease.

Campylobacter pylori

Spiral, campylobacter-like bacteria were observed in close apposition to gastric mucosa in several cases of gastritis and peptic ulcer and successfully cultivated in 1983. They have been named *C. pylori* (formerly *C. pyloridis*). They are short spirals with four unipolar flagella. They become coccoid in old cultures. They grow on moist, chocolate agar or campylobacter media under microaerophilic conditions at 37°C forming small transparent colonies in 3–4 days. They produce oxidase, catalase, phosphatase and H_2S. A distinctive feature is the formation of abundant urease and this property has been used as a rapid diagnostic test in gastric biopsy samples. They do not metabolise carbohydrates or reduce nitrate. They are sensitive to many antimicrobials including penicillin, erythromycin, tetracycline, gentamicin, metronidazole, as well as to bismuth subnitrate. They are resistant to cotrimoxazole and nalidixic acid.

Infection with *C. pylori* causes an acute gastritis which may become chronic. It is commonly associated with peptic ulcer, but its aetiological role is yet to be proven.

LEGIONELLA PNEUMOPHILA

The name Legionnaire's disease was given to an apparently new illness which broke out among members of the American Legion who attended a convention in Philadelphia in 1976. The disease was characterised by fever, cough and chest pain, leading on to pneumonia and often ending fatally. The causative agent has been called *Legionella pneumophila*. Subsequent investigations have revealed that the disease is neither new nor localised. Infection with *L. pneumophila* is now known to cause protean manifestations. Two distinct clinical patterns have been identified and designated Legionnaire's disease and Pontiac fever.

Legionnaire's disease may be either epidemic or sporadic. The illness is characterised by fever, nonproductive cough and dyspnoea. Diarrhoea and encephalopathy are common. Case fatality may be 15–20 per cent, the cause of death being progressive respiratory failure and shock. All ages are susceptible, though more cases have occurred in the elderly. Smoking and alcohol are risk factors.

Pontiac fever is a milder, nonfatal illness with fever, chills, myalgia and headache.

L. pneumophila is a short Gram negative, nonmotile rod with fastidious growth requirements. It was originally isolated by inoculation into guinea pigs and yolk sac of embryonated eggs. It can be grown in the FG (Feeley-Gorman) medium, consisting of Mueller-Hinton agar, ferric pyrophosphate and L cysteine. It is an aerobe, growing optimally at 35°C and at pH 6.9. Colonies are tiny and appear in 4–10 days. It is catalase positive and weakly oxidase positive. Sugar fermentation, urease and nitrate tests are negative. No fewer than 23 Legionella species are now recognised. *L. pneumophila* is classified into 10 serogroups, the majority of human infections being caused by serogroup 1.

Diagnosis can be made by the demonstration of the bacillus by immunofluorescence, by isolation in culture or by serology. The bacillus stains well by Dieterle's silver impregnation method.

Infection is airborne. The source of infection has frequently been traced to airconditioning units. The bacillus is present in soil and water as parasites on free living amoebae. It is sensitive to most antibiotics, except vancomycin. Erythromycin and rifampin have been reported to be useful in treatment.

Several other fastidious bacteria have been isolated in recent years from cases of severe pneumonia. These have been collectively termed atypical Legionella-like organisms (A.L.L.O).

EIKENELLA CORRODENS

This is an oxidase positive, facultatively anaerobic, capnophilic, Gram negative bacillus. The name 'corrodens' refers to the characteristic pitting or corroding of blood agar by colonies of the bacterium. It is present in the mouth, upper respiratory tract and gastrointestinal tract of humans. Infection follows salivary or faecal contamination and usually involves the skin and subcutaneous tissues, though rarely osteomyelitis, pneumonia, endocarditis and meningitis may occur. It is sensitive to penicillin and tetracyclin.

CARDIOBACTERIUM HOMINIS

This Gram negative, pleomorphic bacillus which occurs commonly as a commensal in the human nose and throat may cause endocarditis, particularly in those with preexisting cardiovascular disease. It grows on blood agar under 3–5% CO_2 and high humidity. It ferments a wide range of sugars, forms indole and is oxidase positive, but catalase and nitrate negative. It is sensitive to many antibiotics, penicillin and streptomycin being the recommended drugs.

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46 Rickettsiaceae

The family Rickettsiaceae contains microorganisms causing typhus fevers and related diseases Because of their obligate intracellular parasitism and inability to grow in cell free cultures, they were formerly considered closely related to viruses. But they are Gram negative pleomorphic rods dividing by binary fission and possessing a cell wall containing muramic acid, metabolic enzymes independent of the host cell, and both DNA and RNA. They are large enough to be seen under the light microscope, are held back by bacterial filters and are susceptible to antibiotics They are, therefore, true bacteria, specially adapted to obligate intracellular parasitism. They are primarily parasitic in arthropods such as fleas, lice, ticks and mites, in which they are found in the alimentary canal. The name rickettsia has been given in honour of Ricketts who first observed these microorganisms in Rocky Mountain spotted fever (1909) and who died of typhus fever contracted during the course of his work on the organism.

Morphology: In smears from infected tissues, rickettsiae appear as pleomorphic coccobacilli, 0.3–0.6μ x 0.8–2.0μ in size. They are nonmotile and noncapsulated. They are Gram negative, though they do not take the stain well. They stain bluish purple with Giemsa and Castaneda stains and deep red with Machiavello and Gimenez stains.

Under the electron microscope, the rickettsiae are seen to have a three-layered cell wall and a trilaminar plasma membrane thus resembling Gram negative bacteria. Ribosome-like particles and intracytoplasmic organelles have been observed in the granular cytoplasm. *R. prowazekii* has an amorphous capsule surrounding it.

Cultivation: Rickettsiae are unable to grow in cell free media. *Ro. quintana*, the causative agent of trench fever is an exception and has been found to grow on blood agar. Growth generally occurs in the cytoplasm of infected cells, but in the case of the spotted fever rickettsiae, growth may take place in the nucleus as well. Rickettsiae grow best in cells that are not metabolising actively. The optimum temperature for growth is 32°–35°C.

They are readily cultivated in the yolk sac of developing chick embryos, as first shown by Cox Limited growth occurs on the chorioallantoic membrane. They also grow on mouse fibroblast, HeLa, HEp 2, Detroit 6 and other continuous cell cell lines, but tissue cultures are not satisfactory for primary isolation. Laboratory animals such as guinea pigs and mice are useful for the isolation of rickettsiae from patients. They may also be propagated in arthropods.

Resistance: Rickettsiae are readily inactivated by physical and chemical agents. They are rapidly destroyed at 56°C and at room temperature when separated from host components, unless preserved in skimmed milk or a suspending medium containing sucrose, potassium phosphate and glutamate (SPG medium).

Rickettsiae are susceptible to tetracycline and chloramphenicol. Penicillin and sulphonamides

are ineffective, but paraaminobenzoic acid has inhibitory action on rickettsiae. Sulphonamides may actually enhance the growth of rickettsiae and worsen the condition if administered to patients.

Antigenic structure: At least three types of antigens have been demonstrated. The first is a group specific soluble antigen, present on the surface of rickettsiae. This can be separated from the organisms by repeated washing and centrifugation. The second antigen is associated with the bodies of the rickettsiae. This is species specific and, in the case of scrub typhus, strain specific. The third antigen is an alkali stable polysaccharide found in some rickettsiae and in some strains of *Proteus* bacilli. This sharing of antigens between rickettsiae and *Proteus* is the basis for the Weil-Felix reaction used for the diagnosis of rickettsial infections, by the demonstration of agglutinins to *Proteus* strains OX 19, OX 2 and OX K.

Pathogenesis: Rickettsiae are generally transmitted to man by arthropod vectors, except for Q fever which is usually acquired by ingestion or inhalation. On entry into the human body, the rickettsiae multiply locally and enter the blood. They become localised chiefly in the vascular endothelial cells, which enlarge, degenerate and cause thrombus formation, with partial or complete occlusion of the vascular lumen. The overall pathological features of the rickettsial diseases are similar and can be explained by the damage to the vascular endothelium.

Rickettsiae exhibit two kinds of toxicity. They possess an endotoxin that kills mice following intravenous injection. Endotoxic activity is specifically neutralised by antisera. Antigenically, the endotoxins are group specific. The endotoxins of the major groups of rickettsiae (typhus, spotted fever, scrub typhus) are antigenically distinct but cross reactions occur between the endotoxins of the various members within each group. The second toxic activity is haemolysis of sheep and rabbit cells. This appears to be enzymatic in nature and is suppressed by metabolic inhibitors. It is not clear whether the endotoxic and haemolytic activities are relevant in natural disease.

The long survival of rickettsiae in various organs and lymphatic tissues of infected men and animals is a distinctive feature in pathogenesis and is of importance in the epidemiology of some rickettsial diseases.

Classification: Depending on the diseases they produce, the vectors that transmit them, antigenic relationships, growth properties and resistance to physical and chemical agents, rickettsiae are classified into four genera, *Rickettsia*, *Rochalimaea*, *Coxiella* and *Ehrlichia*. The genus Rickettsia contains the agents causing typhus fevers, spotted fevers and scrub typhus. The agent causing trench fever *Ro. quintana* differs from rickettsiae in being usually extracellular in the arthropod host and in being cultivable in cell free media such as blood agar. It has, therefore, recently been separated into a new genus, *Rochalimaea*, named after da Rocha-Lima, one of the early investigators of rickettsial diseases. The Q fever agent differs from rickettsiae in being smaller, more resistant to physical and chemical agents and transmissible without arthropod vectors. It is, therefore, placed in a separate genus, *Coxiella*, named after Cox who first isolated the agent from ticks and introduced the technique of yolk sac inoculation for its cultivation. A group of intraleucocytic rickettsiae that infect the humans and many animals has been classified under the genus *Ehrlichia*. The first human pathogen of this genus, *Ehr. sennetsu* was isolated in Japan in 1954 from a patient with infectious mononucleosis syndrome. *Ehr. canis*, naturally occurring in dogs has also been reported to cause human infections.

Table 46.1 lists the rickettsiae of medical importance and their characteristics.

Typhus fever group

This group consists of epidemic typhus, recrudescent typhus (Brill-Zinsser disease) and endemic typhus.

TABLE 46 1
Rickettsial diseases of man

Group	Species	Disease	Vector	Vertebrate reservoir	Geographical distribution
Typhus group	*R prowazekii*	Epidemic typhus	Louse	Man	Worldwide
	-do-	Brill-Zinsser disease	—	Man	America, Europe, Australia
	R mooseri	Endemic typhus	Rat flea	Rat	Worldwide
Spotted fever group	*R. rickettsii*	Rocky Mountain spotted fever	Tick	Rabbit, dog small rodents	N. America
	R. siberica	Siberian tick typhus	"	Wild animals, cattle, birds	USSR, Mongolia
	R. conori	Fievre Boutonneuse	"	Dog, rodents	Mediterranean littoral
	-do-	South African tick typhus	"	" "	S. Africa
	-do-	Kenyan tick typhus		Rodents	Kenya
	-do-	Indian tick typhus	"	? Rodents	India
	R. australis	Queensland tick typhus	"	Bush rodents	N Australia
	R. akari	Rickettsial pox	Gamasid mite	Mouse	USA, USSR
Scrub typhus	*R. tsutsugamushi*	Scrub typhus	Trombiculid mite	Small rodents, birds	East Asia, Pacific Islands, Australia
Trench fever	*Ro. quintana*	Trench fever	Louse	Man	Europe (N Africa, Mexico)
Q fever	*Cox. burnetii*	Q Fever	1 Extrahuman—tick 2 Human—Nil	1? Rodents 2 Cattle, sheep, poultry	Worldwide
Ehrlichia	*Ehr. sennetsu*	Infectious mononucleosis syndrome			Japan

Epidemic typhus (classical typhus, gaol fever) has been one of the great scourges of mankind, occurring in devastating epidemics during times of war and famine. The disease has been reported from all parts of the world, but has been particularly common in Russia and Eastern Europe. Napoleon's retreat from Moscow was forced by typhus fever breaking out among his troops. During 1917–1922, there were some 25 million cases in Russia, with about three million deaths. Lenin is said to have remarked, in reference to the outbreaks of louse borne typhus and relapsing fever rampant during the Russian revolution, that 'either socialism will defeat the louse or the louse will defeat socialism'! In recent times, the main foci have been Eastern Europe, Northern and Eastern Africa. The disease is also present in the mountainous regions of Central and South America, Southern Asia and South Africa. In India, the disease occurs in Kashmir. In 1970–1973, epidemics had occurred in Ethiopia, Burundi and Ruanda.

The causative agent of epidemic typhus is *R. prowazekii,* named after von Prowazek, who had contracted fatal typhus fever while investigating the disease. Man is the only natural vertebrate host. Several animals — guinea pigs, mice, cotton rats and gerbils — may be infected experimentally. Natural infection in flying squirrels has been identified in South Eastern USA and they may act as reservoir hosts, infection being spread by the squirrel louse and flea.

The human body louse, *Pediculus humanus corporis,* is the vector. The head louse may also transmit the infection, but not the pubic louse. The lice become infected by feeding on rickettsiaemic patients. The rickettsiae multiply in the gut of the lice and appear in the faeces in 3–5 days. Lice succumb to the infection within 2–4 weeks, remaining infective till they die. The lethal nature of the infection in the louse suggests that the association between *R. prowazekii* and its vector is relatively recent and not well established. Lice may be transferred from person to person. Being sensitive to temperature changes in the host, they leave the febrile patient or the cooling carcass and parasitise other persons. Lice defaecate while feeding and infection is transmitted when the contaminated louse faeces is rubbed in by scratching through the minute abrasions thus caused. Occasionally, infection may also be transmitted through inhalation of aerial suspensions of dried louse faeces or through the conjunctiva.

The incubation period is 5–21 days. The disease starts with fever and chills and cannot be differentiated clinically from many other acute infectious diseases till the characteristic rash appears on the fourth or fifth day. The rash is macular or maculopapular, fading on pressure at first, and starting on the trunk and spreading over the limbs, but sparing the face, palms and soles. Towards the second week, the patient becomes stuporous and delirious. The name typhus comes from the cloudy state of consciousness in the disease (from *typhos,* meaning smoke or cloud). The case fatality varies from 15–70 per cent and increases with age. In some who recover from the disease, the rickettsiae may remain latent in the lymphoid tissues or organs for years. Such latent infection may, at times, be reactivated leading to recrudescent typhus (Brill-Zinsser disease).

Brill (1898) noticed a mild, sporadic, typhus-like disease in New York, among Jewish immigrants from South Eastern Europe. Zinsser (1934) isolated *R. prowazekii* from such cases and proved that they were recrudescences of infections acquired many years previously. The Brill-Zinsser disease explains the manner in which the rickettsia is able to survive without extrahuman reservoirs. In itself, the disease is not important, but such cases occurring in louse ridden communities may initiate epidemics of typhus fever.

Endemic typhus (murine or flea borne typhus) is a milder disease than epidemic typhus. It is caused by *R. mooseri (R. typhi)* which is maintained in nature as a mild infection of rats, transmitted by the rat flea, *Xenopsylla cheopis.* The rickettsia multiplies in the gut of the flea and is shed in its faeces. The flea is unaffected, but remains infectious for the rest of its natural

of life. Domestic cats and the cat flea, *Ctenocephalides felis,* have also been incriminated in the transmission of endemic typhus.

Man acquires the disease usually through the bite of infected fleas. Ingestion of food recently contaminated with infected rat urine or flea faeces may also cause the disease. Human infection is a dead end. Man to man transmission does not occur. In Kashmir and China, lice have been known to transmit murine typhus in man, producing smouldering outbreaks.

R. mooseri and *R. prowazekii* are closely similar, but may be differentiated by biological and immunological tests. When male guinea pigs are inoculated intraperitoneally with blood from a case of endemic typhus or with a culture of *R. mooseri,* they develop fever and a characteristic scrotal inflammation. The scrotum becomes enlarged and the testes cannot be pushed back into the abdomen because of inflammatory adhesions between the layers of the tunica vaginalis. This is known as the Neill-Mooser or the tunica reaction. Intracytoplasmic rickettsia can be seen in the stained smears of scrapings from the tunica. The Neill-Mooser reaction is negative with *R. prowazekii*. *R. mooseri* can be maintained indefinitely by serial passage in mice, but *R. prowazekii* tends to degenerate. *R. mooseri* causes heavy infection with profuse multiplication when inoculated intranasally or intraperitoneally in rats, but *R. prowazekii* produces only inapparent or mild infection. Following recovery from epidemic or endemic typhus, there is immunity to reinfection with both rickettsiae, but vaccination confers only homologous and not heterologous immunity. The two rickettsiae may be differentiated by complement fixation and rickettsial agglutination tests.

Endemic typhus is of worldwide incidence, but is not of much public health importance as the disease is mild and sporadic and can be easily controlled.

A third member of the typhus fever group is *R. canada,* which was isolated from ticks in Canada. It shares the group antigen with *R. prowazekii* and *R. mooseri,* but can be differentiated by toxin neutralisation and complement fixation tests. It is also related to the spotted fever group of rickettsiae and, like them, grows in the nuclei of infected cells. Its role as a human pathogen has not been established, but there is serological evidence that it may cause a disease resembling spotted fever.

Spotted fever group

Rickettsiae of this group possess a common soluble antigen and multiply in the nucleus as well as the cytoplasm of host cells. They are all transmitted by ticks, except *R. akari,* which is mite borne. Six species have been recognised in this group. *R. rickettsii* is the causative agent of Rocky Mountain spotted fever. *R. siberica* causes Siberian tick typhus, *R. conori* Indian, Mediterranean, Kenyan and South African tick typhus and *R. australis* the Queensland tick typhus, *R. parkeri,* isolated from ticks causes infection in guinea pigs, but has not been identified as a human pathogen. *R. akari* causes rickettsial pox.

Rickettsiae are transmitted transovarially in ticks which, therefore, constitute both vectors and reservoirs. The infection may be transmitted to vertebrate hosts by any of the larval stages or by adult ticks. The ticks are not harmed by the rickettsiae and remain infected for life. The rickettsiae are shed in tick faeces, but transmission to man is primarily by bite, as the rickettsiae invade the salivary glands of the ticks also. All rickettsiae of this group pass through natural cycles in domestic and wild animals or birds.

Rocky Mountain spotted fever is the most serious type of spotted fever and is the first to have been described. It resembles epidemic typhus clinically, but the rash, which is macular initially becoming petechial later, appears first on the flexor aspects of the wrist and ankle and then spreads all over the body, including the palms, soles and even the buccal mucosa. The case fatality is variable, but has been 18 per cent overall in the USA. It was originally identified in Montana and Idaho (Northwest USA), where it was shown

to be transmitted by the wood tick, *Dermacentor andersoni*. Subsequently, the disease was observed in the eastern parts of the USA also, where the vector was the dog tick, *D. variabilis*.

The Siberian tick typhus is a mild rickettsial disease occurring in Siberia, America and the Central Asian republics of the USSR. The disease is transmitted by several species of ixodid ticks. The causative agent, *R. siberica* is closely related to *R. rickettsii*. Both these species cause a severe scrotal reaction in guinea pigs, leading to scrotal necrosis and death.

Tick typhus in several parts of Europe, Africa and Asia is caused by *R. conori*, strains of which isolated from Mediterranean littoral, Kenya, South Africa and India are indistinguishable. The species is named after Conor, who provided the first description of the Mediterranean disease, fievre boutonneuse (1910). The disease was first observed in India by Megaw (1917) in the foothills of the Himalayas. The investigations of Kalra, Rao, Soman, Helig and Naidu have established that the disease is found in many parts of India. The tick *Rhipicephalus sanguineus* is the most important vector. *Haemaphysalis leachi*, *Amblyomma* and *Hyalomma* ticks can also transmit the infection.

R. australis, the causative agent of Australian (Queensland) tick typhus is closely related to *R. conori* and is transmitted by ixodid ticks.

Rickettsial pox, the mildest rickettsial disease of man is a self-limited, nonfatal, vesicular exanthem first observed in New York (1946) The name is derived from the resemblance of the disease to chickenpox. It is also called vesicular or varicelliform rickettsiosis. The causative agent is *R. akari* (from *akari*, meaning mite). The reservoir of infection is the domestic mouse, *Mus musculus* and the vector is the mite, *Allodermanyssus sanguineus*, in which transovarial transmission occurs. *R. akari* has also been isolated from wild rodents in Korea. The disease has been reported from Russia also.

R. conori, *R. australis* and *R. akari* cause fever and scrotal swelling in guinea pigs, similar to the Neill-Mooser reaction caused by *R. mooseri*.

Scrub typhus

Scrub typhus is caused by *R. tsutsugamushi* (*R. orientalis*) and occurs all along East Asia, from Korea to Indonesia, and in the Pacific islands, including Australia. It was first observed in Japan, where it was found to be transmitted by mites. The disease was, therefore, called *tsutsugamushi* (from *tsutsuga*, meaning dangerous and *mushi*, meaning insect or mite). It is a place disease and is found only in areas with a suitable climate, plenty of moisture and scrub vegetation. The vector mite is *Trombicula akamushi* in Japan and *T. deliensis* in India. The mites inhabit sharply demarcated areas in the soil where the microecosystem is favourable (mite islands). Man is infected when he trespasses into these mite islands and is bitten by the mite larvae (chiggers). The mite feeds on the serum of warmblooded animals only once during its cycle of development, and adult mites feed only on plants. Rickettsiae are transmitted transovarially in mites. Various rodents and birds act as reservoirs and also help in spreading the rickettsiae to fresh areas.

Scrub typhus was originally believed to be confined to scrub jungles. But it has also been found to occur in a variety of other habitats, such as sandy beaches, mountain deserts, and equatorial rain forests. The term 'chigger borne typhus' has therefore been suggested as a more apt designation. Four factors are essential for the establishment of a microfocus of infection, viz. coexistence and intimate relationship among *R. tsutsugamushi*, chiggers, rats and secondary or transitional forms of vegetation (known as the 'zoonotic tetrad').

The incubation period is 1–3 weeks. Most patients develop a characteristic eschar at the site of the mite bite. The disease sets in with fever, headache and conjunctival injection. A red macular rash appears on the fifth day. Case fatality rates are highly variable, ranging from 0.5 per cent to 60 per cent. The disease is not a serious

problem in civilian practice, but assumes very great importance in military medicine during jungle warfare, as was recognised in the Indo-Burmese theatre in the Second World War.

Considerable differences exist among different strains of *R. tsutsugamushi* in antigenic properties and virulence, a factor that complicates serodiagnosis and immunoprophylaxis. Three major antigenic types have been recognised — Karp, Gilliam and Kato. The rickettsia is more pathogenic to mice than to guinea pigs.

Trench fever

During the First World War, over a million cases of a disease known as trench fever or five day fever occurred among soldiers fighting in the trenches in Europe. The disease was not fatal, but because of its slow course and prolonged convalescence, it caused a very considerable loss of manpower.

Trench fever is an exclusively human disease and no animal reservoir is known. It is transmitted by the body louse. The faeces of lice becomes infectious 5–10 days after an infectious meal. The lice are unharmed and remain infective throughout their lives. Vertical transmission does not occur in lice. The causative agent was identified as a rickettsia and named *Ro. quintana* (from *quintana*, meaning fifth, referring to the five-day fever, a synonym for trench fever). The agent differs from rickettsiae in the following respects: 1) It occurs extracellularly in the arthropod host and is demonstrable in large masses in the gut lumen of infected lice; 2) it grows poorly in the yolk sac of chick embryos; 3) it does not cause experimental infection in any common laboratory animal. Besides man and the louse, only monkeys can be infected, 4) it is able to grow in bacteriological media such as blood agar. On aerobic incubation at 37°C under increased CO_2 tension, colonies appear after two weeks in primary isolation and 3–5 days in subsequent passages; and 5) convalescent sera from patients do not react with rickettsial or *Proteus* antigens. These differences have led to *Ro. quintana* being separated into a new genus in the tribe Rickettsieae, designated *Rochalimaea quintana*.

The disease frequently lead to chronic rickettsiaemia, *Ro. quinatana* having been isolated from the blood of patients upto two years after the acute attack. Recrudescence may occur as in the Brill-Zinsser disease and relapses have been reported as long as 20 years after the primary disease. The chronic rickettsiaemia and late relapses help to maintain the rickettsia in the absence of animal reservoirs.

Trench fever is of little concern now. The only known epidemics occurred during the two World Wars. Due to the mild clinical picture and difficulties in laboratory diagnosis, sporadic cases, if at all they occur, are not likely to be identified. Isolation of *Ro. quintana* from Tunisia and Mexico recently, suggest that the disease may be more widely distributed than is realised.

Q fever

Derrick (1935) investigated cases of fever occurring in abattoir workers in Brisbane, Australia, and transmitted the infection to guinea pigs by inoculation of blood from patients. As the aetiology of the disease was unknown, it was referred to as 'Query' or *Q fever*. Burnet identified the causative agent as a rickettsia and it was named *R. burnetii*. At about the same time the same agent was isolated, independently in the USA from ticks by Cox and was designated *R. diaporica*, the name referring to its ability to pass through filters impermeable to other rickettsiae. The Australian and the American isolates were subsequently shown to be identical. As the Q fever agent exhibits several peculiarities, it has been separated from other rickettsiae into a special genus and has been named *Coxiella burnetii*.

Q fever is the most widely distributed of all rickettsial diseases and has been reported from virtually all countries. It is primarily a zoonosis, solidly established in domestic livestock the world over. Wild animals such as the bandicoot may constitute the primary reservoir, the infection being transmitted among them by ixodid ticks. Trans-

ovarial transmission has been demonstrated in ticks. The rickettsiae are abundant in tick faeces and survive in them for long periods in the dry state. Ticks transmit the disease to cattle, sheep and poultry. The rickettsiae are shed in the milk of infected cattle. They are particularly abundant in the conception products of infected animals and contaminates the environment at parturition. Human infections have been traced to consumption of infected milk, handling of infected wool or hides, soil contaminated with the faeces of infected animals, infected straw and contaminated clothing. Thus, it is an occupational disease. The infecting organism may enter through the abraded skin, lungs, mucous membrane and gastrointestinal tract. Though man to man transmission has been reported, it is very rare. Ticks do not play any significant role in transmitting the infection to man.

Cox. burnetii appears to be widely distributed in India. Though very few human cases have been documented, serological surveys in different areas have demonstrated high prevalence of antibodies by complement fixation and capillary agglutination tests. The infection appears to be prevalent among many animals including the sheep, goats, cattle, buffaloes, dogs, fowl, pigs, mules and camels. The rickettsia has been isolated from cow's milk and ticks.

The human disease is an acute systemic infection, usually characterised by an interstitial pneumonia. The clinical picture is very variable and asymptomatic infections are very common. No rash occurs in the disease. The rickettsiae spread through almost all the organs and frequently affect the liver and heart leading to hepatitis and subacute bacterial endocarditis. Spontaneous recovery usually occurs. The rickettsia may remain latent in the tissues of patients for 2–3 years. Man to man transmission is rare.

Cox. burnetii is pleomorphic, occurring as small rods 0.2–0.4μ x 0.4–1.0μ in size or as spheres 0.3μ – 0.4 μ in diameter. It is filterable. Generally regarded as Gram negative, it appears Gram positive when alcoholic iodine is used as the mordant. It is relatively resistant to physical and chemical agents. In dried tick faeces and in wool, it survives for a year or more at 4°C and in meat for at least a month. It is not completely inactivated at 60°C or by 1% phenol, in one hour. Milk may not be freed of the infective agent by pasteurisation by the holding method, but the flash method is effective. It grows well in the yolk sac of chick embryos and in various types of cell cultures. Ether extraction of yolk sac cultures does not release a soluble antigen as in the case of other rickettsiae. *Cox. burnetii* is the only rickettsia to exhibit phase variation. Fresh isolates are in phase I. They become phase II on repeated passage in yolk sac, but reversion to phase I takes place by passaging in guinea pigs. Phase II cells are autoagglutinable and are phagocytosed in the absence of specific antibody. Phase I activity is attributed to a surface carbohydrate antigen which is soluble in trichloracetic acid and is destroyed by periodate. Phase I is a more powerful immunogen than Phase II and elicits antibodies in high titre to both antigens. Phase II antigen gives better results in complement fixation tests with human and animal sera. Q fever sera do not react with other rickettsial antigens or with *Proteus*.

Laboratory diagnosis of rickettsial diseases: Rickettsial diseases may be diagnosed in the laboratory either by isolation of the rickettsiae or by serology. As rickettsiae are highly infectious and have caused several serious and fatal infections among laboratory workers, their isolation should be attempted with utmost care and only in laboratories equipped with appropriate safety provisions.

Rickettsiae may be isolated in male guinea pigs or mice from patients in the early phase of the disease. Blood clot ground in skimmed milk or any suitable suspending medium is inoculated intraperitoneally. In Q fever, besides blood, the sputum and less often the urine, may yield the causative agent. The animals have to be observed for 3–4 weeks and their temperature recorded daily. Their response to rickettsial infection varies. In Rocky Mountain spotted fever, guinea

pigs develop fever, scrotal necrosis and may even die. With *R. mooseri*, *R. conori* and *R. akari* they develop fever and tunica reaction. *R. prowazekii* and *Cox. burnetii* produce only fever without any testicular inflammation. Mice are preferable to guinea pigs for the isolation of *R. tsutsugamushi*. Infected mice become ill and develop ascitis. Smears from peritoneum, tunica and spleen of infected animals may be stained by Giemsa or Gimenez methods to demonstrate the rickettsiae.

Though laboratory strains of rickettsiae grow profusely in the yolk sac of chick embryos, this method and tissue culture are not suitable for primary isolation.

Ro. quintana will not grow in guinea pigs or mice. It can be isolated by letting healthy lice feed on the patient. The rickettsia may be demonstrated in the gut of such lice. This method is known as xenodiagnosis. It may also be cultivated from the patient's blood on blood agar or in liquid media containing fetal calf serum.

Serological diagnosis may be by the heterophile Weil-Felix reaction or by specific tests using rickettsial antigens. The Weil-Felix reaction is an agglutination test in which sera are tested for agglutinins to the O antigens of certain nonmotile Proteus strains OX 19, OX 2 and OX K. The test was developed from the chance observation of Weil and Felix (1916) that a Proteus strain isolated from the urine of a patient of epidemic typhus was agglutinated by the patient's serum as well as by the sera of other typhus patients. The basis of the test is the sharing of an alkali stable carbohydrate antigen by some rickettsiae and by certain strains of Proteus, *P. vulgaris* OX 19, and OX 2 and *P. mirabilis* OX K. The test is usually done as a tube agglutination, though rapid slide agglutination methods have been employed for screening.

Sera from epidemic and endemic typhus agglutinate OX 19 and sometimes OX 2 also. The test is negative or only weakly positive in Brill-Zinsser disease. In tick borne spotted fever both OX 19 and OX 2 are agglutinated. OX K agglutinins are found only in scrub typhus. The test is negative in rickettsial pox, trench fever and Q fever (Table 46.2)

The Weil-Felix reaction is a simple and useful test for the diagnosis of some rickettsial diseases. The antibody appears rapidly during the course of the disease, reaches peak titres of upto 1:1000 or 1:5000 by the second week and declines rapidly during convalescence. The test is of no value in the diagnosis of rickettsial pox, trench fever and Q fever. It may also be negative in about 75 per

TABLE 46.2
Weil-Felix reaction in rickettsial diseases

Disease	Agglutination pattern with		
	OX 19	OX 2	OX K
Epidemic typhus	+++	±	–
Brill-Zinsser disease	Usually negative or weak positive		–
Endemic typhus	+++	±	–
Tick borne spotted fever	++	++	–
Rickettsial pox	–	–	–
Scrub typhus	–	–	+++
Trench fever	–	–	–
Q fever	–	–	–

cent of recrudescent typhus and 50 per cent of scrub typhus cases. It does not differentiate between epidemic and endemic typhus. False positive reaction may occur in some cases of urinary or other infections by Proteus and at times in typhoid fever and liver diseases. Hence it is desirable to demonstrate a rise in titre of antibodies for the diagnosis of rickettsial infections.

The most frequently used serological method using rickettsial antigens is the complement fixation test. This may be done using the group specific soluble antigen or the type specific washed rickettsial antigen The former test is in routine use, but the latter is necessary for differentiation between epidemic and endemic typhus Due to the high degree of heterogeneity in *R. tsutsugamushi*, it is necessary to use as antigens, representative strains such as Gilliam, Karp and Kato. In Q fever, it is important to use phase II antigens for CF test, the usual antigens being prepared from the Henzerling (Italian) and Nine Mile (USA) strains. A satisfactory CF antigen for trench fever has been prepared from *Ro quintana* cultured on blood agar

Other serological tests include agglutination of rickettsial suspensions, passive haemagglutination of red cells sensitised by ESS (erythrocyte sensitising substance), toxin neutralisation, immunofluorescence and radioisotope precipitation. A skin test has been developed for demonstration of delayed hypersensitivity to *Cox. burnetii* A positive skin test is considered to be a contraindication for Q fever vaccination

Immmunoprophylaxis of rickettsial diseases. Rickettsial diseases may be prevented by general measures such as control of vectors and animal reservoirs. Control of Q fever requires adequate pasteurisation of milk and care in the handling of animals and their products Immunisation is useful in special situations.

Killed and live vaccines have been prepared against epidemic typhus. The earliest of these was phenolised intestinal contents of lice infected per rectum with *R. prowazekii* (Weigl's vaccine). This was too complicated for mass production. Castaneda developed a formalinised mouse lung vaccine. Effective mass vaccination became possible only after Cox developed the inactivated yolk sac vaccine. A live vaccine using the attenuated strain E has been found to be highly immunogenic, but a proportion of vaccinees develop mild disease.

The Cox type vaccine confers effective protection against Rocky Mountain spotted fever. Immunoprophylaxis in scrub typhus is rendered difficult by the antigenic heterogeneity of *R. tsutsugamushi* In Q fever, killed *Cox. burnetii* vaccine causes severe local reaction. Purified antigens are under investigation and a trichloracetic acid extract from purified phase I suspensions of *Cox. burnetii* promises to be very effective in preventing Q fever in man

BARTONELLA BACILLIFORMIS

This is a pleomorphic, Gram negative, motile, rod shaped organism that causes two distinct clinical conditions — a fatal febrile anaemia called Oroya fever and a benign nodular skin lesion called Verruga peruana The organism is seen inside the erythrocytes of infected persons. It can be cultivated in semisolid agar with fresh rabbit or human blood. Inoculation of cultures into monkeys leads to Verruga peruana, but not Oroya fever, except rarely and in atypical form. The disease is confined to the mountainous areas of the American Andes in tropical Peru, Columbia and Ecuador.

B. bacilliformis has been classified under the family Bartonellaceae in the order Rickettsiales.

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47 Chlamydiae

A group of intracellular microorganisms causing psittacosis, lymphogranuloma venereum and trachoma in man and diverse diseases in many avian and mammalian species has been for long the object of taxonomical confusion. As they are filterable and do not grow in cell free media, they were considered to be viruses. They were called psittacosis-lymphogranuloma-trachoma (PLT) viruses. As they produce basophilic intracytoplasmic inclusion bodies in infected cells, in contrast to the eosinophilic inclusions produced by most viruses, they were sometimes referred to as 'basophilic viruses'. They have since been shown to differ from viruses in several properties. They possess cell walls as in bacteria and rickettsiae, do not have an 'eclipse phase' following cellular infection, multiply by binary fission, possess both DNA and RNA and are susceptible to antibiotics and chemotherapeutic agents. They have, therefore, been considered to occupy a position intermediate between rickettsiae and viruses. Following the suggestion of Moulder, it is now generally agreed that they be considered as small bacteria adapted to obligate intracellular parasitism. Because of the phylogenetic uncertainty, the noncommittal term 'agent' was often used to refer to these microorganisms.

Several names have been proposed for the PLT group. In recognition of the pioneering studies of Sir Samuel Bedson on the psittacosis agent, the name Bedsoniae was proposed for the group. Though this term was very popular, the official term for this group now is Chlamydia. The name Chlamydia is derived from the characteristic appearance of the inclusion bodies produced by these agents, which are seen enclosing the nuclei of infected cells as a cloak or mantle (*chlamys*, meaning mantle).

Chlamydiae are classified into two species: *Chlam. psittaci* and *Chlam. trachomatis*. *Chlam. trachomatis* strains form compact inclusions with glycogen matrix, are sensitive to sulphonamides and are natural parasites of humans causing usually localised infections of the eye and genitals. *Chlam. psittaci* strains form diffuse vacuolated inclusions without a glycogen matrix, are resistant to sulphonamides and are natural parasites of birds and animals, capable of causing pneumonia and generalised infections in humans.

Morphology: Chlamydiae occur in two forms, elementary bodies and reticulate (initial) bodies. The elmentary body is a spherical particle, 200–300 nm in diameter with an electron dense nucleoid. This is the extracellular infectious particle. On entry into a suitable host cell, it enlarges to form the reticulate body, 500 – 1000 nm in size. This is the reproductive form and divides by binary fission to produce a large number of elementary bodies (Fig. 47.1).

Chlamydiae are Gram negative, but they stain better with Castaneda, Machiavello or Gimenez stains. The inclusion bodies are basophilic. The inclusion bodies of *Chlam. trachomatis* can be stained with Lugol's iodine because of the glycogen matrix they possess.

General properties of chlamydiae: Chylamydiae are heat labile, being inactivated within minutes at 56°C. They are susceptible to ethanol, ether

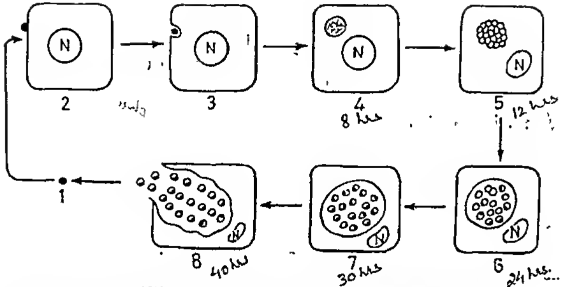

Fig 47.1 Reproductive cycle of Chlamydiae.
1. Elementary Body (EB); 2 EB attaches to cell receptor, 3 EB enters cell by phagocytosis; 4 8 hours EB reorganised into large infectious Reticulate Body (RB), 5 12 hours. Host cell growth arrested. RB undergoing fission, 6 24 hours Inclusion Body with RB and developing EB, 7 30 hours Inclusion Body containing infectious EB, Nucleus pushed to periphery, 8 40 hours Death and lysis of cell releasing EB

and low concentrations of phenol and formalin. Infectivity is maintained for several days at 4°C. They can be preserved frozen at –70°C or lyophilised

Chalmydiae can be propagated in the mouse, chick embryo or in tissue culture, though they show individual variations in susceptilibilty.

They possess two types of antigens. All members share a common heat stable, complement fixing antigen. This can be extracted in ether, deoxycholate or chloroform methanol. Chemically, it is a lipid carbohydrate protein complex similar to the cell wall antigens of Gram negative bacteria. Some members possess a soluble haemagglutinin which agglutinates murine and vaccinia sensitive fowl erythrocytes. A heat labile antigen left on the cell wall after extraction with deoxycholate or trypsin is type specific and helps to distinguish the different species or types by complement fixation or neutralisation tests Chlamydiae produce a toxin, probably lipoprotein in nature, which is lethal to mice on intravenous inoculation. The toxin is neutralised specifically by the antitoxin and toxin neutralisation affords a method of intraspecies typing. Typing methods have been developed using micro-immunofluorescence.

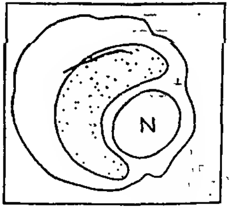

Fig 47.2 Halberstaedter-Prowazek body in trachoma Conjunctival epithelial cell containing a large reniform inclusion body surrounding the nucleus (N)

TABLE 47.1

Human diseases caused by Chlamydiae

Species	Serotype*	Disease
Chlam. trachomatis	A,B,Ba,C.	Hyperendemic blinding trachoma.
Chlam. trachomatis	D,E,F,G,H,I,J,K	Inclusion conjunctivitis (neonatal and adult). Nongonococcal urethritis, cervicitis, salpingitis, epididymitis, proctitis, pneumonia of newborns.
Chlam. trachomatis	L1, L2, L3	Lymphogranuloma venereum
Chlam. psittaci	Many serotypes	Psittacosis
Chlam psittaci	TWAR strain	Human ARD

* *Predominant types associated with the disease.*

Chlam. trachomatis strains have been classified by neutralisation and immunofluorescence tests into 15 serotypes—A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2 and L3. Types A, B, Ba and C, cause endemic trachoma (type A in the Middle East and North Africa, the others worldwide) and are spread from eye to eye. Types D to K cause oculogenital infections (inclusion conjunctivitis, nonspecific urethritis, pelvic infections). The serotypes A to K are together called TRIC agents (from Trachoma-Inclusion-Conjunctivitis). Types L1, L2 and L3 cause the venereal disease LGV. The LGV serotypes cause a lethal infection when inoculated intracerebrally in mice and can spread from cell to cell in susceptible tissue cultures. These properties differentiate them from TRIC agents.

Chlam. psittaci contains many unidentified serotypes. Their interrelationships have not been worked out (Table 47.1). Grayston (1986) has reported a new strain of *Chlam. psittaci* designated TWAR (from Taiwan Acute Respiratory) strain which appears to be a common cause of acute respiratory disease, pneumonia and bronchitis in older children and adults. Antibodies have been demonstrated in the sera of adults from different parts of the world. This appears to be a human strain of *Chlam. psittaci* transmitted from man to man, without any avian or animal host.

PSITTACOSIS

Psittacosis is a disease of parrots and other psittacine birds, transmissible to man (*psittacos*, meaning parrot). A similar but generally milder disease acquired by contact with nonpsittacine birds is called ornithosis (*ornithos*, meaning bird). Psittacosis infection has been recognised in over 70 species of the parrot family, and ornithosis in a large number of other birds. Infection in birds is usually subclinical leading to a carrier state. Overt disease may be precipitated by caging or overcrowding and is manifested as diarrhoea, mucopurulent respiratory discharge and emaciation. The chlamydiae are shed in the droppings or nasal discharge, and aerosols are liberated. Man is infected by inhalation and after an incubation period of about ten days develops clinical disease which may vary from a mild influenza-like syndrome to a fatal pneumonia. Though pneumonia is the usual clinical manifestation, psittacosis is a septicaemia and may occasionally lead to meningoencephalitis, endocarditis, pericarditis, arthritis or a typhoid-like syndrome. Case to case transmission is rare but has been reported. The

high infectivity of psittacosis is indicated by the frequency of laboratory infections. Strains from parrots and turkeys are more virulent than those from other avian species and may cause fatality of upto 20 per cent. *Chlam. psittaci* causes natural infection, often inapparent, in many species of animals, but these strains are of low virulence for humans.

Laboratory diagnosis: The chlamydia can be isolated from the blood during the early stages of the disease and from the sputum later on also. Sputum is treated with streptomycin to suppress contaminants. The material is inoculated into mice intranasally, intraperitoneally or intracerebrally and into the yolk sac of 6–8 day old eggs. The mice die within ten days and smears of the lung, peritoneal exudate, spleen or brain will show the elementary bodies (Levinthal Cole Lillie or LCL bodies). LCL bodies can be seen in the smears of yolk sac from infected eggs as well. Serial passage may sometimes be necessary for isolation. It is generally difficult to recover chlamydia from patients treated with antibiotics. Isolation should be attempted only in laboratories where special care can be taken, as laboratory infection is a serious hazard.

Serological diagnosis may be made by demonstrating rise in titre of CF antibodies in paired serum samples. The antigen is group specific and false positive reactions may be seen in other chlamydial infections. A type specific CF test has been described using live antigen, but technical difficulties preculde its routine use.

Control: Psittacosis has been controlled in some countries by checking the import of birds, but ornithosis is worldwide in distribution and cannot be so controlled.

Treatment: The chlamydia is susceptible to penicillin, tetracycline, erythromycin and chloramphenicol. Tetracycline is the drug of choice, treatment being continued for several days after clinical improvement.

LYMPHOGRANULOMA VENEREUM

This disease characterised by suppurative inguinal adenitis has been known in the tropical countries for a long time under various names, lymphogranuloma inguinale, poradenitis, climatic or tropical bubo. The disease is transmitted venereally. The primary lesion is a small papulovesicular lesion appearing on the external genitalia after an incubation period varying from three days to five weeks. This may at times be extragenital depending on the mode of infection. This is usually painless and may pass unnoticed. The secondary stage developing about two weeks later results from lymphatic spread to the draining lymph nodes. In males the inguinal lymph nodes are involved most often and in females the intrapelvic and pararectal nodes. The nodes enlarge, suppurate, become adherent to the skin and break down to form sinuses discharging pus. Metastatic complications may sometimes occur, with involvement of joints, eyes and meninges. The tertiary stage is chronic, lasting for several years, representing the sequelae of scarring and lymphatic blockage. Late sequelae are more distressing in females leading to rectal strictures and elephantiasis of the vulva (esthiomene).

Laboratory diagnosis: The primary lesion usually goes unnoticed and the disease is seen commonly first in the stage of inguinal adenitis (bubo). Smears of the material aspirated from the bubos may show the elementary bodies (Miyagawa's granulocorpuscles). The material, with streptomycin added, may be inoculated into mice intracerebrally or into the yolk sac of developing eggs. Isolation is difficult.

Serological diagnosis may be made by the group specific CF test, but false positive results may occur in other chlamydial infections. As in psittacosis, a type specific CF antigen has been described, but it is too complicated for routine use.

The method most commonly used for the diagnosis of LGV is the intradermal test described by Frei (1925). As originally described, the antigen

was bubo pus diluted five-fold in saline and heated at 60°C for two hours. This antigen was later replaced by preparations from infected mouse brain, but the frequency of nonspecific reactions made this unsuitable. At present, the antigen is prepared from infected yolk sac, purified by fractional centrifugation and inactivated by phenol or heat. This antigen (Lygranum) is inoculated intradermally on the forearm (0.1 ml) and a control prepared from uninfected yolk sac on the other forearm. A positive reaction is indicated by an inflammatory nodule appearing at the test site in two days and reaching a maximum in 4–5 days, measuring at least 7 mm in diameter. Frei's test becomes positive 2–6 weeks after infection and remains positive for several years, perhaps for life. The antigen is group specific and false positive reactions may occur in other chlamydial infections. A type specific skin test antigen has been developed by acid extraction of the chlamydia, but this is not routinely available.

Treatment: LGV is susceptible to sulphonamides and antibiotics. Tetracycline is the antibiotic commonly employed. Treatment should be prolonged to ensure eradication of the infection. Corticosteriods may be useful in the treatment of old cases with fibrosis.

TRACHOMA AND INCLUSION CONJUNCTIVITIS

Trachoma is a chronic keratoconjunctivitis characterised by follicular hypertrophy, papillary hyperplasia, pannus formation and in the late stages, cicatrisation. Though Halberstaedter and Prowazek in 1907 transmitted the infection to orangutans and demonstrated in conjuctival smears the characteristic inclusion body that bears their names, cultivation of the chlamydia became possible only half a century later, when Tang and his colleagues (1957) grew it in the yolk sac of eggs.

Infection is transmitted from eye to eye by fingers or fomites. Flies may transmit the infection mechanically. It may also be carried by dust, in which case infection may be facilitated by minor abrasions caused by dust particles. The incubation period is variable and influenced by the dose of infection. Onset is insidious.

Trachoma has been classified into several stages. The earliest is trachoma dubium, where the disease is just a suspicion. Protrachoma is the stage of conjunctival lesion before follicles become visible. TRIC agents and inclusions are not usually demonstrable in these early stages. Established trachoma progresses through stages I to IV. Infectivity is maximum in the early cases. Stage IV is noninfectious.

Laboratory diagnosis: The characteristic inclusions (Halberstaedter Prowazek or HP bodies) may be demonstrated in conjunctival scrapings, after staining by Giemsa, Castaneda or Machiavello methods. Because they possess a glycogen matrix they may be stained with iodine solution also. The fluorescent antibody method enhances the sensitivity of smear diagnosis.

The chlamydia may be grown in the yolk sac of 6–8 days old eggs. The material is treated with streptomycin or polymyxin B before inoculation. The eggs are incubated at 35°C in a humid atmosphere. Blind passages may be necessary for isolation. This method is time consuming and cumbersome.

Tissue culture using stationary phase cells (nonreplicating cells) is the method of choice for isolation. McCoy cells rendered nonreplicating by irradiation or antimetabolites are used. HeLa cells treated with DEAE dextran may also be used. In both the cases the inoculum has to be driven into the cells by centrifugation upto 15,000 g to get a good growth.

Complement fixing antibodies appear in the serum, but this test is not generally useful for diagnosis. Type specific antibodies may be demonstrated by microimmunofluorescence.

Treatment: Local application and oral administration of sulphonamides and of tetracycline or other suitable antibiotic should be continued for several weeks. Treatment is most effective in the

D–K

early cases, but relapses may occur. Local steroid application may do harm.

Epidemiology and control: Trachoma is worldwide in distribution and about 500 millinn people are estimated to be affected. It is particularly prevalent in the developing nations because of unhygienic conditions. It is endemic in the Middle East, Africa, India and the Far East. Contrnl of the disease involves mass education and chemotherapy. Vaccination has not proved tn be an effective or practicable method of control.

Inclusion conjunctivitis

The epidemiology nf this condition, first recognised by Halberstaedter and Prowazek in 1910 had to be reestablished in recent years. The natural habitat of *Chlam. trachomatis* types D to K is the genital tract in both sexes.

'Inclusion blennrrhoea', the neonatal form nf inclusion conjunctivitis, develops when the infant is infected in the birth passage. It usually becomes apparent between 5–12 days after birth. It was considered to be benign and self-limited, but has a high incidence of micrnpannus, conjunctival scars and late recurrences. These can be prevented by local application nf tetracycline.

In the adult form of the disease, there is follicular hypertrophy with scanty nonpurulent discharge. It was known as 'swimming pool conjunctivitis' as infection was associated with bathing in community swimming pools which presumably get contaminated with chlamydia from the genital secretions of bathers. Contamination of the eye with the patient's own genital secretion may be the cause more often of 'genital chlamydiasis'.

Genital chlamydiasis

Chlam. trachomatis types D–K are frequently responsible for sexually transmitted diseases. In the female, the infection is mostly asymptomatic, but may cause cervicitis, salpingitis and pelvic inflammation. Symptomatic disease is commoner in the male, with urethritis being the most common complaint ('nonspecific nongonococcal or postgnnococcal urethritis'). It may also cause epididymitis and proctitis. Perinatal infection causes inclusion conjunctivitis and pneumonia in the newborn.

The true prevalence of genital chlamydiasis has not been recognised, particularly in the developing countries due to the difficulties in laboratory diagnosis. Demonstratiun nf inclusion bodies in Giemsa stained smears is a very insensitive method. Cultivation of the chlamydia is possible only in specialised laboratories. Immunofluorescence has been found to be a useful diagnostic method. Direct immunofluorescence test using labelled monoclonal antibodies is highly specific and sensitive. Enzyme-immunoassays and DNA probes have been introduced for diagnosis of chlamydial infections.

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48 General Properties of Viruses

Unicellular microorganisms may be classified in the descending order of complexity as the eukaryotes, such as protozoa and fungi, and the prokaryotes, such as bacteria, mycoplasmas, rickettsiae and chlamydiae. Viruses do not fall strictly into the category of unicellular microorganisms as they do not possess a cellular organisation. Even the simplest of microorganisms are cells enclosed within a cell wall, containing both types of nucleic acid (DNA and RNA), synthesising their own macromolecular constituents and multiplying by binary fission. Viruses, on the other hand, do not have a cellular organisation. They contain only one type of nucleic acid, either DNA or RNA, but never both. They are obligate intracellular parasites. They lack the enzymes necessary for protein and nucleic acid synthesis and are dependent for replication, on the synthetic machinery of host cells. They multiply by a complex process and not by binary fission. They are unaffected by antibacterial antibiotics. The major differences between viruses and microorganisms are shown in Table 48.1. In spite of these basic differences, viruses are generally considered as microorganisms in medical microbiology.

Viruses occupy the twilight zone that separates the 'living' from the 'nonliving'. The demonstration by Stanley (1935) that viruses could be crystallised like chemicals, and the extraction by Gierer and Schramm (1956) of 'infectious nucleic acid' from a virus that could infect host cells and yield complete virus progeny, made it appear that viruses were only 'living chemicals'. Recent advances in molecular biology seem to make the distinction between 'life' and 'nonlife' little more than a semantic exercise. As the smallest 'living units', viruses offer the best models for understanding the chemistry of 'life'.

The medical importance of viruses lies in their ability to cause a very large number of human diseases. Viral diseases range from minor ailments such as the common cold to highly fatal diseases such as rabies or yellow fever. They may be sporadic such as mumps, endemic such as rabies, epidemic such as measles or pandemic such as influenza. They may be localised to circumscribed areas (as some arbovirus diseases) or worldwide (as Herpes simplex). The control of bacterial infections with antibiotics has enhanced the role of virus infections in human disease. Viruses can cause cancer in animals and birds, and in human beings as well.

Morphology of viruses

Size: The extracellular infectious virus particle is called the *virion*. Viruses are much smaller than bacteria. It was their small size and 'filterability' (ability to pass through filters that would hold back bacteria) that led to their recognition as a separate class of infectious agents. Hence they were for a time known as 'filterable viruses'. As they were too small to be seen under the light microscope, they were called 'ultramicroscopic'. Some of the larger viruses, such as poxviruses, can be seen under the light microscope when suitably stained. The virus particles seen in this manner are known as elementary bodies.

Viruses vary widely in size. The largest among

TABLE 48.1

Properties of prokaryotes and viruses

	Cellular organisation	Growth on inanimate media	Binary fission	Both DNA and RNA	Ribosomes	Sensitivity to antibacterial antibiotics	Sensitivity to interferon
Bacteria	+	+	+	+	+	+	−
Mycoplasmas	+	+	+	+	+	+	−
Rickettsiae	+	∓	+	+	+	+	−
Chlamydiae	+	−	+	+	+	+	+
Viruses	−	−	−	−	−	−	+

them (e.g. poxviruses) measuring about 300 nm, are as large as the smallest bacteria (mycoplasma). The smallest viruses (e.g. foot and mouth disease virus) measuring about 20 nm are nearly as small as the largest protein molecules such as haemocyanin.

The earliest method for estimating the size of virus particles was by passing them through collodion membrane filters of graded porosity (gradocol membranes). The average pore diameter of the finest filter that permitted passage of the virion gave an estimate of its size. With the development of the ultracentrifuge, a second method became available. From the rate of sedimentation of virus in the ultracentrifuge, the particle size could be calculated using the Stokes' law. The third and the most direct method for measuring virus size is electron microscopy. Purified preparations of virions may be examined under the electron microscope either unstained or stained. By this method, the shape as well as the size of virions can be studied.

Structure and shape: The virion consists essentially of a nucleic acid core surrounded by a protein coat, the *capsid*. The capsid with the enclosed nucleic acid is known as the *nucleocapsid*. The function of the capsid is to protect the nucleic acid from inactivation by nucleases and other deleterious agents in the environment. The capsid is composed of a large number of *capsomers* which form its morphological units. The chemical units of the capsid are polypeptide molecules which are arranged symmetrically to form an impenetrable shell around the nucleic acid core (Fig. 48.1). One of the major functions of the capsid is to introduce viral genome into host cells by adsorbing readily to cell surfaces.

Two kinds of symmetry are met with in the capsid — icosahedral (cubical) and helical. An icosahedron is a polygon with 12 vertices or corners and 20 facets or sides. Each facet is in the shape of an equilateral triangle. Two types of capsomers constitute the icosahedral capsid. They are the pentagonal capsomers at the vertices (pentons) and the hexagonal capsomers making up the facets (hexons). There are always 12 pentons but the number of hexons varies with the virus group. In nucleocapsids with helical symmetry, the capsomers and nucleic acid are wound together to form a helical or spiral tube. The tube may be rigid, as in the tobacco mosaic virus, but in the case of animal viruses, the tubular nucleocapsid is pliable and may be coiled on itself. Not all viruses show the typical icosahedral or helical symmetry. Some, like the poxviruses, exhibit a complex symmetry.

Virions may be enveloped or nonenveloped (naked). The envelope or outer covering of viruses is derived from the host cell membrane when the progeny virus is released by budding. The envelope is lipoprotein in nature. The lipid is largely of host cell origin while the protein is virus

helical symmetry.

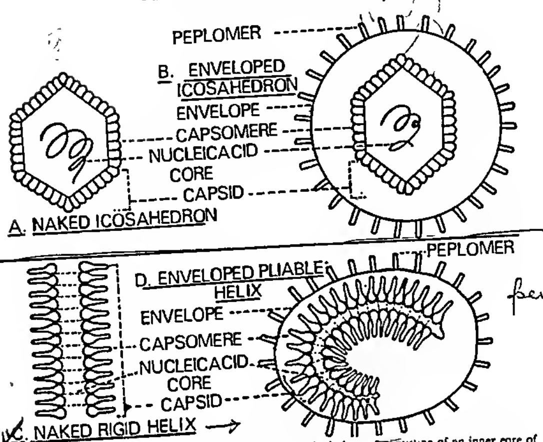

Fig 48.1 Design and structure of virions. A—naked icosahedral virus, consisting of an inner core of nucleic acid enclosed by a capsid, which is made of capsomers B—differs from A in possessing an envelope C—naked helical virus composed of capsomers wound round the nucleic acid to form a tubular structure. D—enveloped helical virus in which the tubular capsid is pliable and is enclosed within an envelope

coded. Protein subunits may be seen as projecting spikes on the surface of the envelope. These structures are called *peplomers* (from *peplos*, meaning envelope). A virus may have more than one type of peplomer. Thus the influenza virus carries two kinds of peplomers — the haemagglutinin which is a triangular spike and the neuraminidase which is a mushroom shaped structure. Envelopes confer chemical, antigenic and biological properties on viruses. Enveloped viruses are susceptible to the action of lipid solvents like ether, chloroform and bile salts. Specific neutralisation of virus infectivity depends on antibodies to the surface antigens. Biological properties such as haemagglutination depend on the envelope. Some viruses possess additional structural features. For example, fibrils protrude from the vertices of adenovirus particles.

The overall shape of the virus particle varies in different groups of viruses. Most animal viruses are roughly spherical. Some are irregular and pleomorphic. The rabies virus is bullet shaped and poxviruses are brickshaped. The tobacco mosaic virus is rod shaped. Bacterial viruses have a complex morphology (Fig. 48.2).

Chemical properties: Viruses contain only one type of nucleic acid, either single or double stranded DNA or RNA. In this respect, viruses are unique, for nowhere else in nature is genetic information solely carried by RNA. Viral nucleic acids may be extracted by treatment with detergents or phenol and, in the case of some viruses e.g., picornavirus, papovavirus), the extracted nucleic acid is capable of initiating infection when introduced into host cells.

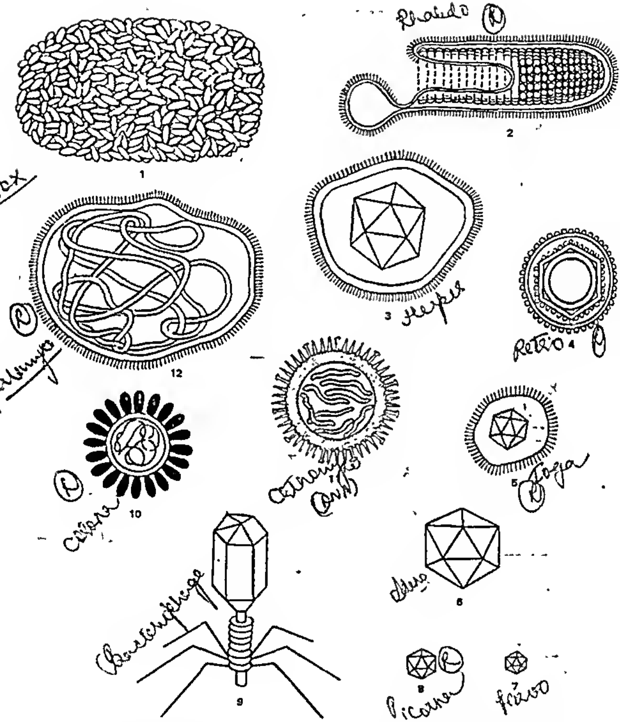

Fig. 48.2 Comparative sizes and shapes of different groups of viruses 1 Poxvirus 2, Rhabdovirus 3 Herpesvirus 4 Retrovirus 5 Togavirus 6 Adenovirus 7, Parvovirus 8 Picornavirus 9 Bacteriophage 10 Coronavirus 11 Orthomyxovirus 12 Paramyxovirus

Viruses also contain protein which makes up the capsid. Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the virus. Enveloped viruses contain lipids derived from the host cell membrane. Some viruses also contain small amounts of carbohydrate. Most viruses do not possess any enzymes for synthesis of viral components or for energy production, but some have other enzymes, as for example, the neuraminidase in the influenza virus.

Resistance: With few exceptions, viruses are very heat labile. There are individual variations, but in general, they are inactivated within seconds at 56°C, minutes at 37° and days at 4°C. They are stable at low temperatures. For long term storage, they are kept frozen at −70°C. A better method for prolonged storage is lyophilisation or freeze drying (drying the frozen virus under vacuum). Lyophilised virus can be stored indefinitely and can be reconstituted when required by addition of water. Some viruses (e.g., poliovirus) do not stand freeze drying. Viruses vary greatly in their resistance to acidity. For example, enteroviruses are very resistant to acid pH while rhinoviruses are very susceptible. All viruses are disrupted under alkaline conditions.

Viruses are inactivated by sunlight, UV rays and ionising radiations. They are, in general, more resistant than bacteria to chemical disinfectants, probably because they lack enzymes. Phenolic disinfectants are only weakly virucidal. Bacteria are killed in 50 per cent glycerol saline, but this acts as a preservative for many viruses (e.g., vaccinia, rabies). Molar concentrations of certain salts ($MgCl_2$, Na_2SO_4) also protect some viruses (e.g., poliovirus) against heat inactivation. The most active antiviral disinfectants are oxidising agents such as hydrogen peroxide, potassium permanganate and hypochlorites. Organic iodine compounds are actively virucidal. Chlorination of drinking water kills most viruses, but its efficacy is greatly influenced by the presence of organic matter. Some viruses (e.g., hepatitis virus, polioviruses) are relatively resistant to chlorination. Formaldehyde and beta propiolactone are actively virucidal and are commonly employed for the preparation of killed viral vaccines.

The action of lipid solvents such as ether, chloroform and bile salts is selective, the enveloped viruses being sensitive and the naked viruses resistant to them. This selective action is useful in the identification and classification of viruses.

Antibiotics active against bacteria are completely ineffective against viruses. This property is made use of in eliminating bacteria from clinical specimens by antibiotic treatment before virus isolation.

Viral haemagglutination

Viral haemagglutination was originally observed with *influenza virus* by *Hirst (1941)*. A large number of viruses have since been shown to agglutinate erythrocytes from different species. Haemagglutination by the influenza virus is due to the presence of haemagglutinin spikes on the surface of the virus. The influenza virus also carries on its surface another peplomer, the enzyme neuraminidase which acts on the receptor and destroys it. Neuraminidase is, therefore, called the 'receptor destroying enzyme' (RDE). RDE is produced by many bacteria including cholera vibrios, and is also present in many vertebrate cells. Destruction of the receptor leads to the reversal of haemagglutination and the release of the virus from the red cell surface. This is known as elution.

Haemagglutination serves as a convenient method for detection and assay of influenza virus. When red cells are added to serial dilutions of a viral suspension, the highest dilution that produces haemagglutination provides the haemagglutination titre. Haemagglutination test can be carried out in test tubes or special plastic trays. Red cells which are not agglutinated settle at the bottom in the form of a 'button', while the agglutinated cells are seen spread into a shield-like pattern (Fig 48.2a). As inactivated virus can

also haemagglutinate, the test serves to titrate killed influenza vaccines. As haemagglutination is specifically inhibited by the antibody to the virus, haemagglutination inhibition provides a convenient test for the antiviral antibody. Haemagglutination and elution also help in purifying and concentrating the virus.

Elution is found only in the myxoviruses that possess neutraminidase. With other viruses, haemagglutination is stable. In the case of arboviruses, haemagglutination appears to be a reversible state of equilibrium between the virus and erythrocytes, being influenced by slight varitions in pH and temperature. Poxviruses agglutinate red cells from only 50 per cent of fowls. The haemagglutinin of poxvirus is distinct from the virion and can be separated by centrifugation. Table 48.2 shows the characteristics of haemagglutination by different viruses.

Viral multiplication

The genetic information necessary for viral replication is contained in the viral nucleic acid, but lacking biosynthetic enzymes, the virus depends on the synthetic machinery of the host cell for replication. Early studies on viral replication employed the bacteriophage as the model. While there are general similarities in the pattern of multiplication of bacterial and animals viruses, there are also important differences. The viral multiplication cycle can be divided into six sequential phases, though the phases may sometimes be overlapping: 1) adsorption or attachment, 2) penetration, 3) uncoating, 4) biosynthesis, 5) maturation, and 6) release.

1. Adsorption: Virions may come into contact with cells by random collision, but adsorption takes place only if there is an affinity between the two. The cell surface should contain specific receptor sites to which the virus can gain attachment. In the case of influenza viruses, the haemagglutinin on the virus surface gets attached to glycoprotein receptor sites on the surface of respiratory epithelium. Destruction of the receptor sites by RDE prevents viral adsorption. In the case of polioviruses, the receptor is the lipoprotein present on the surface of primate, but not rodent cells. The poliovirus can, therefore, attach itself to primate cells, but not in rodent cells. Differences in susceptibility to virus infection are to a large extent based on the presence or absence of receptors on cells. If the phase of adsorption can be bypassed, cells normally insusceptible to a virus may be rendered susceptible to it. Thus,

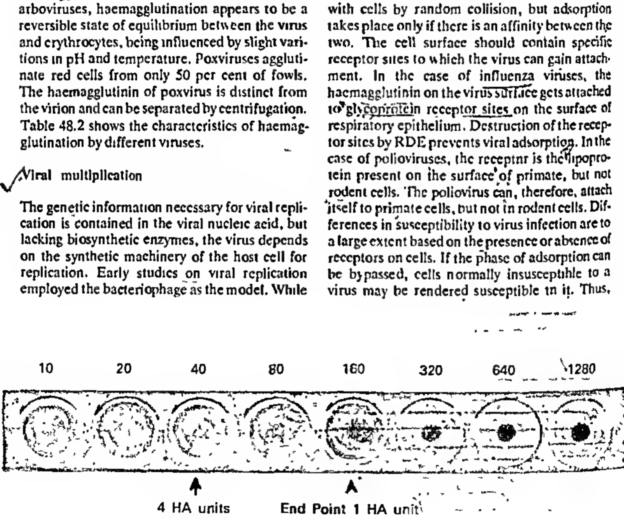

Fig 48.2a Viral haemagglutination. Virus containing fluid is diluted in doubling dilutions and 0.5% suspension of chick red cells added. Where virus is present there is diffuse widespread even pattern on the bottom of the wells in the plastic plate. Where no virus is present the cells settle down to a button-like aggregate with sharp edge.

TABLE 48.2
Characteristics of haemagglutination by viruses

Virus	*Erythrocyte species and other conditions*
Influenza virus	Fowl, human, guinea pig, others; Elution at 37°C
Parainfluenza, mumps, NDV	Fowl, human, guinea pig, others; Elution at 37°C; Haemolysin present
Measles	Monkey, 37°C
Togavirus—several groups of Arbovirus	Goose, pigeon, one day old chick; pH and temperature critical
Rubella	Goose, pigeon, one day old chick, 4°C
Enterovirus—some Coxsaekie and ECHO serotypes	Human, 4°C and 37°C
Rhinovirus, some serotypes	Sheep, 4°C
Rabies	Goose, 4°C, pH 6.2
Reovirus	Human, 37°C
Adenovirus	Monkey, rat; Four groups recognised based on HA of red cells from monkey, rat, both or neither
Poxvirus—smallpox, vaccinia	RBC from some fowls only, Lipoprotein haemagglutinin, not part of virion
Polyomavirus	Guinea pig, 4°C

infectious nucleic acid extracted from picornaviruses can infect rodent cells, which are resistant to infection by the whole virus.

2. *Penetration*: Bacteria possess rigid cell walls. Bacterial viruses cannot, therefore, penetrate into bacterial cells and only the nucleic acid is introduced intracellularly by a complex mechanism. Animal cells do not have rigid cell walls and the whole virus can enter into them. Virus particles may be engulfed by a mechanism resembling phagocytosis, a process known as 'viropexis'. Alternatively, in the case of the enveloped viruses, the viral envelope may fuse with the plasma membrane of the host cell and release the nucleocapsid into the cytoplasm.

3. *Uncoating*: This is the process of stripping the virus of its outer layers and capsid so that the nucleic acid is released into the cell. With most viruses, uncoating is effected by the action of lysosomal enzymes of the host cell. In the poxvirus, uncoating is a two-step process. In the first step, the outer coat is removed by lysosomal enzymes in the phagocytic vacuole. The inner core of the virus, containing the internal protein and nucleic acid, is released into the cytoplasm, where the second step of uncoating is effected by a viral uncoating enzyme and the DNA is liberated.

4. *Biosynthesis*: This phase includes synthesis not merely of the viral nucleic acid and capsid

protein, but also of enzymes necessary in the various stages of viral synthesis, assembly and release. In addition, certain 'regulator proteins' also are synthesised which serve to shut down the normal cellular metabolism and direct the sequential production of viral components. The site of viral synthesis depends on the type of virus. In general, most DNA viruses synthesise their nucleic acid in the host cell nucleus. The exceptions are the poxviruses, which synthesise all their components in the host cell cytoplasm. Most RNA viruses synthesise all their components in the cytoplasm. Exceptions are orthomyxoviruses and some paramyxoviruses and retroviruses which are synthesised partly in the nucleus. Viral protein is synthesised only in the cytoplasm.

Biosynthesis consists essentially of the following steps

1. Transcription of messenger RNA (mRNA) from the viral nucleic acid
2. Translation of the mRNA into 'early proteins'. These 'early or nonstructuraal proteins' are enzymes which initiate and maintain synthesis of virus components. They may also induce shutdown of host protein and nucleic acid synthesis
3. Replication of viral nucleic acid
4. Synthesis of 'late' or structural proteins, which are the components of daughter virion capsids

The mechanisms of transcription and nucleic acid synthesis differ in the different types of viruses. Viral nucleic acids replicate by the Watson-Crick base pairing. In the case of single stranded nucleic acids, a complementary strand is first synthesised, producing double stranded 'replicative forms'. Double stranded viral DNA acts as a template for its replication, and also for transcribing into mRNA the genetic information necessary for the synthesis of capsid proteins and enzymes required in the replicative cycle. RNA viruses use various methods for replication. In poliovirus and many other single stranded RNA viruses, the viral RNA can act directly as mRNA. The single stranded parental RNA (positive strand) acts as the template for the productinn of a complementary strand (negative strand), which in turn acts as the template for progeny viral RNA. In some other single stranded RNA viruses (e.g., influenza virus), parental RNA produces complementary negative strands which act both as mRNA and as template for the synthesis of progeny viral RNA. Oncogenic RNA viruses (retrovirus) exhibit a unique replicative cycle. The virus genome is single stranded RNA. This is converted into an RNA:DNA hybrid by the viral enzyme, RNA directed DNA polymerase (reverse transcriptase). Double stranded DNA is then synthesised from the RNA:DNA hybrid. The double stranded DNA form of the virus (provirus) is integrated into the host cell genome. The provirus acts as the template for the synthesis of progeny viral RNA. The integration of the provirus with the host cell genome may lead to transformation of the cell and development of neoplasia.

5. *Maturation*. Assembly of daughter virions follows the synthesis of viral nucleic acid and proteins. Virion assembly may take place in the host cell nucleus or cytoplasm. Herpes and adenoviruses are assembled in the nucleus while picorna and poxviruses are assembled in the cytoplasm. At this stage, the nonenveloped viruses are present intracellularly as fully developed virions, but in the case of enveloped viruses, only the nucleocapsid is complete. Envelopes are derived from the host cell membrane during the process of budding. The host cell membrane which becomes the envelope is modified by incorporation of virus specific antigens. Herpes viruses assembled in the nucleus acquire their envelope from the nuclear membrane as they are released into the cytoplasm enclosed in a vesicle. Myxoviruses bud from the cell surface and their envelope is formed by the modified cytoplasmic membrane of the host cell. The incorporation of viral antigen (haemagglutinin) on the cell membrane endows the cell with the property of haemadsorption.

6. *Release*. In the case of bacterial viruses, the

release of progeny virions takes place by the lysis of the infected bacterium. But in the case of animal viruses, release usually occurs without cell lysis. Myxoviruses are released by a process of budding from the cell membrane over a period of time. The host cell is unaffected and may even divide, the daughter cells continuing to release virions. Progeny virions are released into the surrounding medium and may infect other cells. In the case of some viruses (e.g., varicella), transmission occurs directly from cell to cell, very little free virus being demonstrable extracellularly in the medium. Not all animal viruses spare the host cell. The poliovirus causes profound damage to the host cell and may be released by cell lysis.

From the stage of penetration till the appearance of mature daughter virions, the virus cannot be demonstrated inside the host cell. This period during which the virus seems to disappear or go 'underground' is known as the 'eclipse phase'. The time taken for a single cycle of replication is about 15–30 minutes for bacteriophages and about 15–30 hours for animal viruses. A single infected cell may release a large number of progeny virions. While this can be determined readily in bacteriophages (burst size), it is difficult to assess in the case of animal viruses that are released over a prolonged period.

Abnormal replicative cycles

A proportion of daughter virions that are produced may not be infective. This is due to defective assembly. Such 'incomplete viruses' are seen in large proportions when cells are infected with a high dose of influenza virus. The virus yield will have a high haemagglutinin titre, but low infectivity. This is known as the *von Magnus phenomenon*.

Virus infection in some cells does not lead to production of infectious progeny. In such cells (nonpermissive cells), the viral components may be synthesised, but maturation or assembly is defective, and either no release occurs, or the progeny is noninfectious. This is known as *abortive infection*. Here the defect is in the type of cell and not in the parental viruses.

Some viruses are generally defective in that when they infect cells, they are unable to give rise to fully formed progeny. Yield of progeny virions occurs only if the cells are simultaneously infected with a helper virus, which can supplement the genetic deficiency. For example, some strains of Rous sarcoma virus (RSV) cannot code for the synthesis of the viral envelope. When RSV infects a cell that harbours a helper virus (e.g., avian leukosis virus), infectious progeny results, the helper virus contributing to the synthesis of the envelope. The envelope antigen of progeny RSV will, therefore, be determined by the type of helper virus. Viruses which are genetically deficient and, therefore, incapable of producing infectious daughter virions are known as 'defective viruses'.

Cultivation of viruses

As viruses are obligate intracellular parasites, they cannot be grown on any inanimate culture medium. Three methods are employed for cultivation of viruses — inoculation into animals, embryonated eggs or tissue cultures.

Animal inoculation: The earliest method for the cultivation of viruses causing human diseases was inoculation into human volunteers. Reed and colleagues (1900) used human volunteers for their pioneering work on yellow fever. Due to the serious risk involved, human volunteers are used only when no other method is available and when the virus is relatively harmless. Monkeys were used for the isolation of the poliovirus by Landsteiner and Popper (1909). But due to their cost and risk to handlers, monkeys find only limited application in virology. In some instances, as in infectious hepatitis, nonhuman primates provide the only method for virus cultivation. The use of white mice, pioneered by Theiler (1903) extended the scope of animal inoculation greatly. Mice are still the most widely employed animals in virology. Infant (suckling) mice are very

ceptible to coxsackie and arboviruses, many of which do not grow in any other system. Mice may be inoculated by several routes — intracerebral, subcutaneous, intraperitoneal or intranasal. Other animals such as guinea pigs, rabbits and ferrets are used in some situations.

The growth of the virus in inoculated animals may be indicated by death, disease or visible lesions. Serial blind passages may sometimes be necessary before evidence of virus growth can be obtained. Disadvantages of animal inoculation are that immunity may interfere with viral growth and that animals often harbour latent viruses. Animal inoculation is also used for the study of pathogenesis, immune response, epidemiology and oncogenesis.

Embryonated eggs: The embryonated hen's egg was first used for the cultivation of viruses by Goodpasture (1931) and the method was further developed by Burnet. The embryonated egg offers several sites for the cultivation of viruses (Fig. 48.3) Inoculation on the chorioallantoic membrane (CAM) produces visible lesions (pocks). Different viruses have different pock morphology. Under optimal conditions, each infectious virus particle can form one pock. Pock counting, therefore, can be used for the assay of pock forming viruses such as variola or vaccinia. Inoculation into the allantoic cavity provides a rich yield of influenza and some paramyxoviruses. Inoculation into the amniotic sac is employed for the primary isolation of influenza virus. Yolk sac inoculation is used for the cultivation of some viruses, chlamydiae and rickettsiae.

Allantoic inoculation is employed for growing influenza virus for vaccine production. Other chick embryo vaccines in routine use are the yellow fever (17D strain) and rabies (Flury strain) vaccines. Duck eggs are bigger and have a longer incubation period than hen's eggs. Therefore, they provide a better yield of rabies virus and are used for the preparation of the inactivated nonneural rabies vaccine.

Cell culture: Cultivation of bits of tissues and organs *in vitro* had been used by physiologists and surgeons for the study of morphogenesis and wound healing. Probably, the first application of tissue culture in virology was by Steinhardt and colleagues (1913) who maintained the vaccinia virus in fragments of rabbit cornea. Maitland (1928) used chopped tissues in nutrient media for cultivation of vaccinia virus. The major obstacle to the development of tissue culture was the presence of bacterial contamination. It was only when antibiotics became available for the prevention of bacterial contamination that tissue culture became a routine laboratory method. The turning point which made tissue culture the most important method for the cultivation of viruses was the demonstration by Enders, Weller and Robbins (1949) that poliovirus, till then considered a strictly neurotropic virus, could be grown in tissue culture of nonneural origin. Since then almost every human virus has been grown in tissue culture.

Three types of tissue cultures are available:

1. Organ culture: Small bits of organs can be maintained *in vitro* for days and weeks, preserving their original architecture and function. Organ cultures are useful for the isolation of some viruses which appear to be highly specialised parasites of certain organs. For example, the tracheal ring organ culture is employed for the isolation of the coronavirus; a respiratory pathogen.

2. Explant culture: Fragments of minced tissue can be grown as 'explants' embedded in plasma clots. They may also be cultivated in suspension. This was what was originally known as 'tissue culture'. This method is now seldom employed in virology. Adenoid tissue explant cultures were used for the isolation of adenoviruses.

3. Cell culture: This is the type of culture routinely employed for growing viruses. Tissues are dissociated into the component cells by the action of proteolytic enzymes such as trypsin and mechanical shaking. The cells are washed, counted and suspended in a growth medium. The essential constituents of the growth medium are: physiologic amounts of 13 essential aminoacids

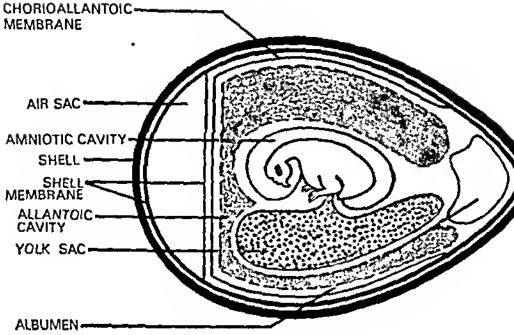

Fig. 48.3 Cross-section of a ten-day-old egg

and nine vitamins, salts, glucose, a buffering system generally consisting of bicarbonate in equilibrium with atmosphere containing about 5% CO_2. This is supplemented with upto 5% calf or fetal calf serum. Antibiotics are added to prevent the growth of contaminants and phenol red as indicator. This medium or one with other ingredients added will enable most cell types to multiply with a division time of 24–48 hours. The cell suspension is dispensed in bottles, tubes or Petri dishes. The cells adhere to the glass surface and, on incubation, divide to form a confluent monolayer sheet of cells covering the surface within about a week.

Cell culture tubes may be incubated in a sloped horizontal position, either as 'stationary culture' or may be rolled in special 'roller drums' to provide better aeration. Some fastidious viruses grow only in such roller cultures.

Based on their origin, chromosomal characters and the number of generations through which they can be maintained, cell cultures are classified into three types (Table 48.3).

a. Primary cell cultures: These are normal cells freshly taken from the body and cultured. They are capable of only limited growth in culture and cannot be maintained in serial culture. Common examples of primary cell cultures are monkey kidney, human embryonic kidney, human amnion and chick embryo cell cultures. Primary cell cultures are useful for the isolation of viruses and their cultivation for vaccine production.

b. Diploid cell strains: These are cells of a single type that retain the original diploid chromosome number and karyotype during serial subcultivation for a limited number of times. After about fifty serial passages, they undergo 'senescence'. Diploid strains developed from human fibroblasts are susceptible to a wide range of human viruses and are very useful for the isolation of some fastidious pathogens. They are also employed for the production of viral vaccines.

c. Continuous cell lines: These are cells of a single type, usually derived from cancer cells, that are capable of continuous serial cultivation indefinitely. Standard cell lines derived from human

TABLE 48.3

Some cell cultures in common use

a	*Primary cell cultures*	
	1. Rhesus monkey kidney cell culture	
	2. Human amnion cell culture	
	3. Chick embryo fibroblast cell culture	
b	*Diploid cell strains*	
	1. WI-38	Human embryonic lung cell strain
	2. HL-8	Rhesus embryo cell strain
c.	*Continuous cell lines*	
	1. HeLa	Human carcinoma of cervix cell line
	2. HEP-2	Human epithelioma of larynx cell line
	3. KB	Human carcinoma of nasopharynx cell line
	4. McCoy	Human synovial carcinoma cell line
	5. Detroit-6	Sternal marrow cell line
	6. Chang C/I/L/K	Human conjunctiva (C) Intestine (I), Liver (L) and Kidney (K) cell lines
	7. Vero	Vervet monkey kidney cell line
	8. BHK-21	Baby Hamster kidney cell line

cancers, such as HeLa, HEp-2 and KB cell lines have been used in laboratories throughout the world for many years. These cell lines may be maintained by serial subcultivation or stored in the cold (–70°C) for use when necessary. They are not used for the manufacture of viral vaccines for human use as the administration of vaccines grown in cancer cells is not considered safe.

Detecion of virus growth in cell cultures: Virus growth in cell cultures can be detected by the following methods:

1. Cytopathic effect: Many viruses cause morphological changes in cultured cells in which they grow. These changes can be readily observed by microscopic examination of the cultures. These changes are known as 'cytopathic effects' (CPE) and the viruses causing CPE are called 'cytopathogenic viruses'. The CPE produced by different groups of viruses are characteristic and help in the presumptive identification of virus isolates. For example, enteroviruses produce rapid CPE with crenation of cells and degeneration of the entire cell sheet; measles virus produces syncytium formation, herpes virus causes discrete focal degeneration, adenovirus produces large granular clumps resembling bunches of grapes, and SV_{40} produces prominent cytoplasmic vacuolation.

2. Metabolic inhibition: In normal cell cultures, the medium turns acid due to cellular metabolism. When viruses grow in cell cultures, cell metabolism is inhibited and there is no acid production. This can be made out by the colour of the indicator (phenol red) incorporated in the medium.

3. Haemadsorption: When haemagglutinating viruses (such as influenza and parainfluenza viruses) grow in cell cultures, their presence can be indicated by the addition of guinea pig erythrocytes to the cultures. If the viruses are multiplying in the culture, the erythrocytes will adsorb on to the surface of cells. This is known as 'haemadsorption'.

4. Interference: The growth of a noncytopathogenic virus in cell culture can be tested by the subsequent challenge with a known cytopathogenic virus. The growth of the first will inhibit infection by the second virus by interference.

5. Transformation: Tumour forming (oncogenic) viruses induce cell 'transformation' and loss of contact inhibition, so that growth apears in a piled-up fashion producing 'microtumours'.

6. Immunofluorescence: Cells from virus infected cultures can be stained by flurescent conjugated antiserum and examined under the UV-microscope for the presence of virus antigen This gives positive results earlier than other methods and, therefore, finds wide application in diagnostic virology

Viral assays ✓

The virus content of a specimen can be assayed in two ways: either with reference to the total virus particles or with reference to the infectious virions only. Two methods employed for total particle enumeration are electron microscopy and haemagglutination. By simple negative staining the virus particles in a suspension can be counted directly under the electron microscope. The virus suspension can be mixed with a known concentration of latex particles. The ratio between the virus and latex particles under the electron microscope gives an indication of the virus count. With haemagglutinating viruses, a convenient method of quantitation is the determination of 'haemagglutination titres'. Haemagglutination is not a very sensitive indicator of the presence of small amounts of virus particles. Thus, approximately 10^7 influenza virions are required to procude macroscopic agglutination of a convenient quantity of chicken erythrocytes (0.5 ml of 0.5 per cent suspension). But because of its simplicity, haemagglutination affords a very convenient method of virus assay.

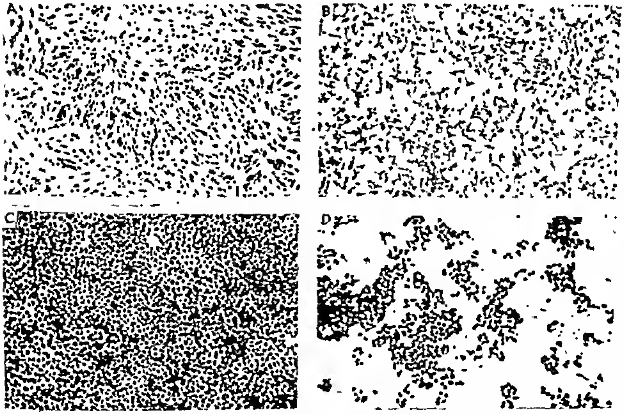

Fig. 48.4A. Normal vero cell monolayer. B. Vero cell monolayer infected with Coxsackie virus B3, stained after 48 hours hours C. Normal HeLa cell monolayer. D HeLa cell monolayer infected with Coxsackie virus B3, stained after 48 hours (Courtesy DR. J. Shanmugam, Sri Chitra Tirunal Medical Centre, Trivandrum)

Assay of infectivity

Two types of infectivity assays can be carried out — quantitative and quantal assays. Quantitative assays measure the actual number of infectious particles in the inoculum while quantal assays only indicate the presence or absence of infectious viruses. By using serial dilutions of virus suspensions and the aid of statisfical methods, reasonably accurate estimates of infectivity can be obtained in quantal assays.

Quantal assays of infectivity can be carried out in animals, eggs or tissue culture. Examples of endpoints used for infectivity titration are the death of the animal, production of haemagglutinin in allantoic fluid or appearance of CPE in cell cultures. The titre is usually expressed as the '50 per cent infectious dose' (ID_{50}) per ml, which indicates the highest dilution of the inoculum that would produce an effect in 50 per cent of animals, eggs or cell cultures inoculated. ID_{50} is calculated by the application of statistical methods, such as that of Reed and Muench.

The quantitative infectivity assay of viruses is similar to the estimation of bacterial viable counts by colony counting. Two methods are available — plaque assay in monolayer cell culture and pock assay on chick embryo CAM. Plaque assay was introduced in animal virology by Dulbecco (1952) as a modification of the bacteriophage plaque assay. A viral suspension is added to a monolayer of cultured cells in a bottle or Petri dish, and after allowing time for absorption, the medium is removed and replaced with a solid agar gel, to ensure that the spread of progeny virions is confined to the immediate vicinity of infected cells. In this system, each infectious viral particle gives rise to a localised focus of infected cells that can be seen with the naked eye. Such foci are known as 'plaques' and each plaque indicates an infectious virus (Fig. 48.5). Some viruses which are transmitted directly from cell to cell (e.g., herpesvirus) may form plaques even without an agar overlay. Oncogenic viruses produce cell transformation which can be seen as microtumours. Hence they can be enumerated by the 'transformation assay'.

Viruses that form pocks on CAM (e.g., vaccinia) can be assayed by counting the number of pocks formed on CAM by appropriate inocula of virus. This is known as 'pock assay'.

Viral genetics

Like all other living beings, viruses obey the laws of genetics. Several properties of viruses, such as virulence and antigenicity, that are of great concern to man in the context of infections at the level of the cell, individual and community, are under genetic control. Genetic studies, therefore, may contribute to better understanding of virus host interactions and help in the development of better viral vaccines. Genetic mechanisms such as mutation and selection had been utilised in the past without recognising the biological mechanisms involved. The development of the 'fixed' rabies virus by Pasteur (1885) is a case in point.

The two main mechanisms for genetic modification in viruses are mutation and recombination. In addition, viruses may exhibit many nonheritable variations due to gene product interactions.

Mutation: The frequency of mutation in viruses is about 10^{-4} to 10^{-8}, approximately the same as in bacteria. Mutations, therefore, occur during every viral infection. Most mutations are lethal. *A mutant becomes evident only if the mutation* confers some readily observable property or survival advantage. Mutation may occur spontaneously or may be induced by mutagens, physical agents such as irradiation or chemical agents such as 5-fluorouracil. (5-F.U.)

The mutants may be of various types. Some mutations of clinical and laboratory interest are those affecting virulence, host range, antigenicity and pock or plaque morphology. A class of mutants that are of great importance in laboratory studies is the conditional lethal mutant. These are mutants which are able to grow under certain conditions (called permissive conditions), but cannot grow under certain other specified conditions

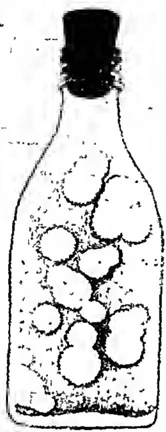

Fig. 48.5 Plaque formation in monkey kidney cells by poliovirus

(called nonpermissive or *restrictive conditions*). There are different types of conditional lethal mutants, but the types most widely employed in genetic studies are the 'temperature sensitive' (*ts*) mutants. These can grow at a low (permissive) temperature (28° to 31°), but not at a higher (restrictive) temperature (37°C). The advantage here is that by using a single selective test (temperature sensitivity), large numbers of mutants with lesions in different genes may be obtained. The *ts* mutants have not only contributed largely to fundamental studies on viral genetics, but also, because of their low virulence, offer prospects of better live viral vaccines.

Recombination: Genetic recombination may occur when two different, but related, viruses infect a cell simultaneously. The two viruses exchange segments of nucleic acid between them so that a hybrid results, possessing genes from both parents. Such recombinants breed true thereafter. Recombinants may occur between 1) two active (infectious) viruses, 2) one active and one inactive virus, and 3) two inactive viruses.

When two different strains of the same virus (such as vaccinia or influenza), possessing distinctive markers (such as pock morphology or antigenic properties) are grown together, recombinants may be derived that possess the distinctive properties of both parents. Thus, if a human and an avian strain of influenza virus (whose haemagglutinin and neuraminidase antigens are different and easily identifiable) are grown together, a hybrid may be obtained with the haemagglutinin of one parent and the neuraminidase of the other. This has been demonstrated experimentally *in vitro* and *in vivo*. This may be one of the ways by which pandemic strains of influenza virus originate in nature.

When a cell is 'infected' with an active virus and a different but related inactive virus, progeny possessing one or more genetic traits of the inactivated virus may be produced. This phenomenon is called *'cross reactivation'* or *'marker rescue'*. New antigenic variants of the influenza virus causing epidemics, often do not grow well in eggs as compared to established laboratory strains. When such an epidemic strain (e.g., strain A_2) is grown in eggs along with a standard strain (e.g., strain A_0) inactivated by UV irradiation, a progeny may be obtained which has the antigenic characters of A_2 but the growth characteristics of A_0. This finds application in the manufacture of the influenza virus vaccines.

When a cell is 'infected' with a large dose (high multiplicity) of a single virus inactivated by UV irradiation, live virus may be produced. The different virions that cause multiple infection of a cell may have suffered damage to different genes so that from the total genetic pool it may be possible to obtain a full complement of undamaged genes. This explains how infectious progeny can be produced. This phenomenon is called *multiplicity reactivation*. There is the potential danger of a multiplicity reactivation taking place follow-

ing the administration of UV irradiated vaccines UV irradiation is, therefore, not acceptable as a method of producing inactivated virus vaccines.

Recombination may take place between virus genome and host chromosome. No viral progeny is produced, but the genetic recombination leads to changes in the host cell, such as malignant transformation.

As a general rule virus capsids enclose viral nucleic acids. Sometimes segments of host nucleic acid become encapsidated instead. For example, in a papovavirus capsid, a linear piece of host DNA roughly the same size as the papovavirus genome may be found. This is known as *pseudovirion*. As far as is known each pseudovirion contains a different piece of host DNA. Generally, pseudovirions make up only a small fraction of the yield. When cells are infected with many virus particles (as in papovavirus), these progeny contain DNA molecules that consist partly of viral and partly of host sequences.

Viral particles containing host DNA sequences are important because of their potential ability to transduce host genes from one cell to another. This could be exploited for correcting inborn errors of metabolism.

Nongenetic Interactions

Phenotypic mixing: When two different viruses multiply in a cell, some 'mix up' may take place during assembly, so that the genome of one virus may be surrounded by a capsid belonging partly or entirely to the other virus. This is known as phenotypic mixing. This is not a stable variation. Upon subsequent passage, the capsid will be found to be of the original type only. In phenotypic mixing, when the nucleic acid of one virus is surrounded by the entire capsid of the other virus, it is known as *transcapsidation*. When phenotypic mixing occurs between two enveloped viruses, resulting in the sharing of peplomers between the two, mosaic envelopes result.

Genotypic mixing or heterozygosis results from the incorporation of more than one complete genome into a single virus particle. There is no recombination between the different genomes so that the two kinds of viral progeny are formed on passage.

Complementation. Complementation is a functional interaction between the gene products (proteins specified by genes) of two viruses, one or both of which may be defective resulting in the multiplication of one or both under conditions in which replication would not ordinarily occur. There is no genetic interaction and the progeny are like parental viruses. A number of different types of complementation may occur. When a rabbit is injected with a mixture of heat inactivated virulent myxomavirus and active avirulent fibroma virus the rabbit develops fatal myxomatosis. Both myxoma and fibroma are poxviruses. Heat inactivated myxoma virus cannot initiate infection because a heat labile enzyme (DNA dependent RNA polymerase) is destroyed. When coinfected with active fibroma virus, it provides the necessary enzyme so that myxoma virus can cause infection.

Tests for complementation between different mutants of a virus provide information about the functional organisation of the viral genome. Such test using *ts* mutants have been very useful in the genetic mapping of viruses.

Interference: The usual result of mixed or multiple infection of cells is interference in which infection of a cell by one virus inhibits simultaneous or subsequent infection by another virus. The most important mediator of interference is 'interferon', a soluble cellular product (described in Chapter 49). Interference may also be produced by destruction of cell receptors by an active or inactive virus, so that subsequent viral attachment is not possible. Such 'viral attachment interference' is seen with myxoviruses and enteroviruses for which cell receptors are important for initiation of infection. Another type of interference is 'autointerference', in which a high multiplicity of infection inhibits production of infectious progeny.

Enhancement: Mixed infection of cells may sometimes lead to increased virus yield or greater CPE. This is known as 'enhancement'. One mechanism of enhancement appears to be suppression of interferon.

Classification and nomenclature of viruses

Till about 1950 little was known of the basic properties of viruses. They were named haphazardly, based on the diseases they caused or on the place of their isolation. They were grouped according to assumed 'tropisms' or affinity to different systems or organs of the body. Thus human viruses were classified as dermotropic, i.e., those producing skin lesions (e.g., smallpox, chicken pox, measles), neurotropic, i.e., those affecting the nervous system (e.g., poliomyelitis, rabies), pneumotropic, i.e., those affecting the respiratory tract (e.g., influenza, common cold) and viscerotropic, i.e., those affecting visceral organs (e.g., yellow fever, hepatitis). Bawden (1941) made the pioneering suggestion that viral nomenclature and classification should be based on the properties of viruses and not upon host responses. From the early 1950's, viruses began to be classified into groups based on their physicochemical and structural features. Nomenclature and classification are now the official responsibility of the International Committee on Taxonomy of Viruses.

Viruses are classified into two main divisions depending on the type of nucleic acid they possess: the riboviruses are those containing RNA and the deoxyriboviruses are those containing DNA. Further classification is based on other properties such as the strandedness of nucleic acid, symmetry of nucleocapsid, presence of envelope, size and shape of virion and number of capsomers. Short descriptions of the major groups of viruses are given below.

DNA viruses

1. Poxviridae family: These are large, brick shaped or ovoid viruses (300 x 240 x 100 nm), with complex structure, having a lipid containing outer coat, one or two lateral bodies and a core carrying a single linear molecule of double stranded DNA. Multiplication and maturation take place in cytoplasm. The family is divided into several genera.

2. Herpesviridae family: These are medium sized viruses containing linear double stranded DNA. The icosahedral nucleocapsid (100 nm) has 162 capsomers and is surrounded by a lipid containing envelope. Multiplication takes place in the nucleus and maturation by budding through the nuclear membrane. Only one genus, *Herpesvirus*, has been characterised, but several members of the family await classification.

3. Adenoviridae family: These are medium sized (70–90 nm) nonenveloped, icosahedral viruses with 252 capsomers. Members have been classified into two genera:

Mastadenovirus (mammalian adenoviruses)
Aviadenovirus (adenoviruses of birds)

4. Papovaviridae family: These are small (40–55 nm) nonenveloped, double stranded DNA viruses with 72 capsomers. Two genera have been recognised.

Papillomavirus (containing the Shope rabbit papilloma virus and related viruses)

Polyomavirus (containing mouse polyomas virus, SV_{40} or vacuolating agent from monkey, B.K. and J.C viruses from man and related viruses)

5. Parvoviridae family: These are very small (18–26 nm) nonenveloped viruses with 32 capsomers. The genome consists of single stranded DNA. Three genera have been described: *Parvovirus*, *Adenosatellovirus* and *Densovirus*.

6. Hepadnaviridae: This consists of the human hepatitis type B virus and related viruses of animals and birds. The name comes from *hepa* = liver, and *dna* for DNA core. The virion is spherical, 42 nm in diameter, consisting of a 27 nm core surrounded by an envelope having virus specific antigens.

ds

RNA viruses

1. *Picornaviridae family* : These are small (20-30 nm) nonenveloped icosahedral viruses with single stranded RNA genome. Two genera have been recognised

Enterovirus, including polio, coxasackie, echo and several other related viruses.

Rhinovirus, including human, bovine and equine rhinoviruses and foot and mouth disease virus.

2. *Orthomyxoviridae family* . These are medium sized (80-120 nm) spherical or elongated enveloped viruses carrying haemagglutinin and neuraminidase peplomers. Genome consists of single stranded RNA in several (8) pieces. Only one genus, *Influenzavirus* has been recognised. Influenzavirus type C possesses several distinctive features and may have to be separated into a new genus.

3. *Paramyxoviridae family* : Pleomorphic virions (150 nm) with lipid envelope, having surface projections. Genome is unsegmented single stranded linear RNA. Three genera have been recognised·

Paramyxovirus, consisting of Newcastle disease virus, mumps virus and parainfluenza viruses of man, other mammals and birds

Morbillivirus, containing measles, canine distemper, rinderpest and related viruses

Pneumovirus, containing respiratory syncytial virus of man and related viruses

4. *Togaviridae family* . These are spherical viruses, 40-70 nm, with lipoprotein envelope and single stranded RNA genome. Most members multiply in arthropods as well as in vertebrates. Four genera have been described

Alpha virus, consisting of viruses formerly classified as group A arhoviruses.

Flavivirus, consisting of viruses formerly classified as Group B arboviruses.

Rubivirus, consisting of rubella virus.

Pestivirus, consisting of mucosal disease virus, hog cholera virus and related viruses.

5. *Bunyaviridae family*: Spherical, enveloped virions, 90 - 100 nm. All are arthropod borne viruses. Only one genus *Bunyavirus* has been recognised, but a large number of groups and subgroups are included in the genus, such as the Bunyamwera group, arbovirus group C, California group and others. Other possible members include, Rift valley fever, the Crimea-Congo haemorrhagic fever group, phlebotomus group and anopheles A and B groups.

6. *Arenaviridae family*: Spherical or pleomorphic viruses, 50-300 nm, containing a number of electron dense ribosome-like particles giving a sandy appearance. (Hence the name; *arena*, meaning sand in Latin) Members are generally rodent parasites causing persistent infection in the natural host, but capable of infecting man rarely, leading to severe haemorrhagic illness. Only one genus *Arenavirus* has been recognised. Species include lymphocytic choriomeningitis virus, Lassa and members of the Tacaribe complex.

7. *Rhabdoviridae family*. Bullet shaped viruses 130-300 nm long and 70 nm wide, with lipoprotein envelope carrying peplomers. Two genera have been recognised.

Vesiculovirus, containing vesicular stomatitis virus, Chandipura virus (isolated from man in India) and related species.

Lyssavirus, containing rabies virus and related viruses such as Lagos bat, Mokola, Duvenhage and others.

Other genera have been suggested to include rhabdoviruses of insects and plants.

8. *Reoviridae family*: Icosahedral, nonenveloped viruses, 60-80 nm in size having double layered capsids. Genome consists of double stranded RNA in 10-12 pieces. Three genera have been recognised.

Reovirus, containing reoviruses from man, other mammals and birds.

Orbivirus, containing several species of arboviruses such as blue tongue virus, African horse sickness virus and Colorado tick fever virus.

Rotavirus including human rotaviruses, calf diarrhoea virus and related agents. Other genera

may have to be defined to include plant and insect viruses belonging to this family.

9. *Coronaviridae family* : Pleomorphic enveloped viruses around 100 nm, with unique club shaped peplomers projecting as a fringe from the surface, resembling the solar corona (hence the name). Only one genus, *Coronavirus* has been recognised. Members include human corona viruses causing upper respiratory disease, avian infectious bronchitis virus, calf neonatal diarrhoea corona virus, murine hepatitis virus and related viruses

10. *Retroviridae* family: (Re = reverse, tr = transcriptase) These are RNA tumour viruses and related agents. Virions are icosahedral about 100 nm. with lipoprotein envelopes. Characteristic biochemical feature is the presence of RNA dependent DNA polymerase (reverse transcriptase) within the virus. Three subfamilies are recognised.

Oncovirinae, the RNA tumour virus group.

Spumivirinae, the foamy virus group (*Spuma* = foam).

Lentivirinae, (*Lenti* = slow) visna and maedi viruses of sheep belonging to the slow virus group.

11. *Caliciviridae* : These are naked spherical particles (35-39 nm) with 32 cup shaped depressions arranged in isosahedral symmetry.

Viroids

ē out extracellular dormant phase protein-free RNA

The term 'viroid' has been introduced by Diener (1971) for a new class of subviral agents characterised by the apparent absence of an extracellular dormant phase (virion) and by a genome much smaller than those of known viruses. The infective agent is a protein-free, low molecular weight RNA resistant to heat and organic solvents, but sensitive to nucleases. First identified in the potato spindle tuber disease, viroids have been shown to cause some other plant diseases also. It is possible that the causative agents of some animal and human diseases may turn out to belong to the class of viroids.

Prion

ē out any detectable nucleic acid

Yet another unconventional, virus-like agent has been described recently (1982). The causative agent of scrapie and Cruetzfeldt-Jacob disease has been shown to be a small particle (MW, 50,000 and probably 4–6 nm diameter), without any detectable nucleic-acid, resistant to heat (90°C for three minutes), UV rays and nucleases, and sensitive to proteases. The name 'prion' has been proposed for this class of proteinaceous infectious particles. It has been suggested that prions may be responsible for the more chronic neurological degenerative diseases of man also.

Further Reading

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49 Virus Host Interactions: Virus Infections

Virus host interactions may be considered at different levels — at the level of the cell, the individual and the community

At the cellular level, virus infection may cause a broad spectrum of effects, ranging from no apparent cellular damage to rapid cell destruction. Some viruses (e g., poliovirus) cause cell death (cytocidal effect) or even lysis (cytolysis). Others may cause cellular proliferation (e.g., molluscum contagiosum) or malignant transformation (e.g., oncogenic viruses). In some instances the virus and host cell enter into a peaceful coexistence, both replicating independently without any cellular injury, a condition known as steady state infection. In tissue culture, virus infection may lead to readily observable cellular changes (cytopathic effects). These may not parallel the changes produced in the infected animal, as in the latter situation infection is influenced by the various defence mechanisms of the body.

Cellular injury may be due to a number of causes. Early or nonstructural viral proteins often cause a shut down of host protein and DNA synthesis. Large amounts of viral macromolecules that accumulate in the infected cell may distort the cellular architecture and exert a toxic effect. The permeability of plasma membranes may be altered, releasing lysosomal enzymes and lead to autolysis.

Many viruses produce alterations in the cytoplasmic membrane of infected cells. Some (e.g., respiratory syncytial virus) cause fusion of adjacent cell membranes, leading to polykaryocytosis or syncytium formation. Virus coded antigens may appear on the surface of infected cells. These antigens may confer new properties on the cells. For example, viral haemagglutinin appears on the surface of cells infected with influenza virus and causes adsorption of erythrocytes to the cell surface (haemadsorption). Virus coded antigens also appear on the surface of cells transformed by oncogenic viruses.

Certain viruses such as measles, mumps, adenoviruses, cytomegalovirus and varicella cause damage to the chromosomes of host cells. Chromatid gaps and breaks in chromosome 17 occur frequently in cells infected with adenovirus types 12 and 31.

The most characteristic histological feature in virus infected cells is the appearnce of inclusion bodies. *Inclusion bodies* are structures with distinct size, shape, location and staining properties that can be demonstrated in virus infected cells under the light microscope. They may be situated in the cytoplasm (e g., poxviruses), nucleus (e g., herpesviruses) or both (e.g., measles virus). They are generally acidophilic and can be seen as pink structures when stained with Giemsa's or eosin-methylene blue stains. Some viruses (e.g., adenovirus) form basophilic inclusions. Demonstration of inclusion bodies helps in the diagnosis of some virus infections. The presence of intracytoplasmic eosinophilic inclusions (Negri bodies) in the brain cells of animals justifies the presumptive diagnosis of rabies. Vaccinia infected cells show rather smaller multiple inclusions known as Guarnieri bodies. Large inclu-

sions (Bollinger bodies) are seen in fowlpox. Inclusion bodies in molluscum contagiosum (molluscum bodies) are very large (20–30μ) and can be readily seen under the low power microscope. Intranuclear inclusion bodies were classified into two types by Cowdry (1934). Cowdry type A inclusions are of variable size and granular appearance (e.g., herpesvirus, yellow fever virus), while type B inclusions are more circumscribed and often multiple (e.g., adenovirus, poliovirus). Inclusion bodies may be crystalline aggregates of virions or made up of virus antigens present at the site of virus synthesis. Some inclusions represent degenerative changes produced by virus infection which confer altered staining properties on the cell.

Pathogenesis of virus infection

Depending on the clinical outcome, virus infections can be classified as inapparent (subclinical) or apparent (clinical or overt) infections. The latter may be acute, subacute or chronic. Some virus infections are characterised by latency. Latent infections are of different types. Recurrent herpes simplex and herpes zoster are examples of latent infections in which clinical manifestations appear after prolonged periods of quiescence during which the viruses remain hidden in the nerve root ganglia. Another type of latent infection is persistent tolerant infection in which the virus is readily demonstrable in the tissues of the host, but neither disease nor immune response develops. The host is immunologically tolerant to the virus as a result of congenital or neonatal infection. Disease sets in when the tolerance is interrupted. The classical example of persistent tolerant infection is lymphocytic choriomeningitis of mice. Another type of latent infection is seen in neurological diseases such as scrapie in the sheep and kuru in man. This is called slowly progressive or slow infections as the incubation period is unusually long. Yet another class of latent infections is infection by oncogenic viruses.

Viruses enter the body through the respiratory tract, the alimentary tract, skin, conjunctiva and the genital tract. Many viruses are transmitted vertically from parent to progeny.

The respiratory tract offers the most important portal of entry for viruses. A large number of viruses are able to infect the cells of the respiratory tract. Some of them multiply locally to initiate a silent local infection which is followed by lymphatic or haematogenous transport to other situations where more extensive multiplication takes place before systemic illness is manifested. Smallpox and chickenpox are examples of such systemic diseases in which the portal of entry is the respiratory tract. Other viruses, such as influenza and rhinoviruses are restricted to the respiratory tract where they multiply and produce local disease. These are known as respiratory viruses.

Next to the respiratory tract, the alimentary tract is the most important route of entry for viruses. But only some viruses are able to establish infection in the intestines. All enveloped viruses are destroyed by bile. Rhinoviruses are inactivated by gastric acidity. Only enteroviruses, adenoviruses, reoviruses, hepatitis viruses and the viruses causing gastroenteritis are able to set up intestinal infection. Some of these (e.g., gastroenteritis viruses) remain confined to the gut causing local disease. Others (e.g., poliovirus) after initial multiplication locally, are transported to other sites for further multiplication and subsequent spread to the target organs.

Of the viruses that enter through the skin, only a few produce local lesions. Papilloma, vaccinia, cowpox, molluscum contagiosum and orf are probably the only viruses that produce dermal lesions at the site of entry. Skin lesions of exanthematous virus diseases are secondary to systemic infection. Viruses enter the skin through abrasions (e.g., papillomavirus), insect bites (e.g., arboviruses) animal bites (e.g., rabies) or injections (e.g., type B hepatitis). Systemic spread occurs through lymphatics or blood. Rabies virus travels along the nerves to the spinal cord or brain.

Conjunctiva also may act as a portal of entry for viruses. This may lead to local disease (e.g., adenovirus) or to systemic spread (e.g.,

measles). Some viruses may enter through the genital tract or other sites of sexual contact (e.g., HIV).

Congenital infection may occur at any stage from the development of the ovum upto birth. In acute systemic infection (e.g., smallpox), congenital infection usually leads to fetal death and abortion. Rubella and cytomegalovirus produce maldevelopment or severe neonatal disease. Immunological tolerance is not established in human congenital infections, in contrast to lymphocytic choriomeningitis in mice. Vertical transmission is the natural mode of spread of many tumour viruses. The avian leukosis virus is transmitted in ovo and murine mammary tumour virus through breast milk.

Spread of virus in the body

The manner in which the infecting virus spreads from the point of entry, multiplies in sites of election and causes lesions in target tissues was first studied by Fenner (1948) using mousepox as the experimental model (Fig. 49.1). The mousepox virus enters the skin, where it multiplies initially and proceeds along the lymphatics to the local nodes. After multiplication in the lymph nodes, the virus enters the bloodstream (primary viraemia) and is transported to the spleen and liver which act as the 'central foci' for viral multiplication. After extensive multiplication in the central foci, there occurs a massive spillover of the virus into the bloodstream (secondary viraemia). This heralds the onset of clinical symptoms (the prodromal phase in eruptive fevers). The virus reaches the target organ (skin in eruptive fevers) through the bloodstream. Multiplication in the target sites produces the distinctive lesions. With minor modifications, this model holds good for most systemic virus diseases. The reasons for the difference in foci of multiplication and target organs in the case of different viruses are obscure.

Significance of the incubation period

The incubation period represents the time taken for the virus to spread from the site of entry to the organs of viral multiplication and thence to the target organs for the production of lesions. Its duration is, therefore, influenced by the relation between the sites of entry, multiplication and lesions. Where the site of entry and site of lesion are the same, the incubation period is short — one to three days as in respiratory virus infections and in gastroenteritis. In systemic diseases where the virus enters through the respiratory or alimentary tract and produces lesions in remote target sites, the incubation period is long — 10–20 days, as in smallpox or chickenpox. There are, however, exceptions to this rule. In arbovirus diseases, as in yellow fever or dengue, the incubation period may be shorter (5–6 days), probably because the virus is introduced directly into the bloodstream by the insect vectors. Papillomas and molluscum contagiosum have long incubation periods, probably because the viruses multiply slowly. The reason for the unusually prolonged incubation period of slow virus infections is not known.

Host responses to virus infections

The outcome of a virus infection is influenced by the virulence of the infecting strain and the resistance offered by the host. Mechanisms of host resistance may be immunological or nonspecific. The latter includes various genetic and physiological factors such as interferon production, body *temperature, nutrition and hormones.*

Immunity in virus infections: Virions in general are good antigens and induce both humoral and cellular immune responses. The multiplication of a virus in the body during infection induces not only a quantitatively greater immune response, but also liberates and makes available to the immune system the whole range of virus antigens, including surface and internal antigens as well as the nonstructural antigens such as early proteins.

In mediating humoral antiviral immunity, the important classes of antibodies are IgG, IgM and

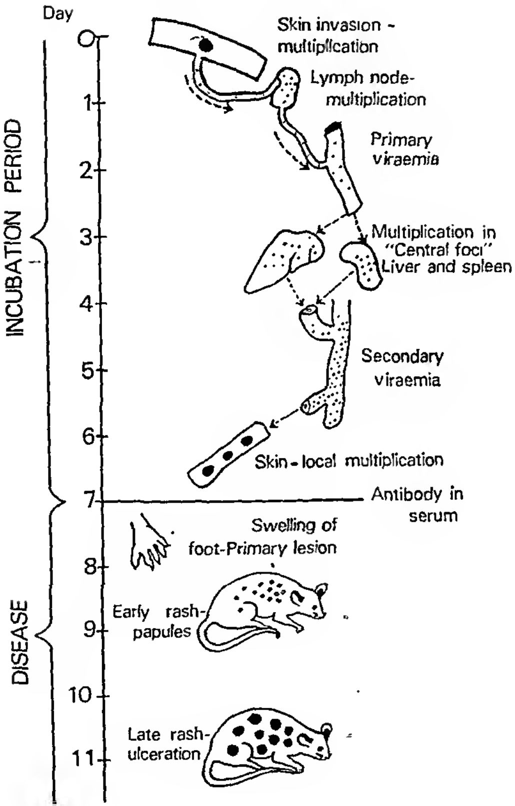

Fig. 49.1 Pathogenesis of mousepox—a model for acute exanthemata of man (After Fenner)

GAM

IgA. IgG and IgM play a major role in blood and tissue spaces while IgA is more important on mucosal surfaces. Antibodies effect virus neutralisation by several mechanisms. They may prevent adsorption of the virus to cell receptors, cause enhanced virus degradation or prevent release of the progeny virus from infected cells. Complement may act in conjunction with antibodies in causing surface damage to enveloped virions and in producing cytolysis of virus infected cells.

Not all antibodies are able to neutralise virus infectivity. Antibodies to internal antigens are non-neutralising. Antibodies to surface antigens vary in their neutralising ability. For instance, two types of surface antibodies appear following influenza infection — antihaemagglutinin and antineuraminidase. The former can neutralise infectivity, but the latter cannot. Antineuraminidase antibody can, however, inhibit the release of progeny virions from infected cells. Some antibodies can paradoxically enhance virus infectivity. Humoral antibodies may sometimes actually contribute to pathogenesis. Antibodies may cause complement dependent injury to cells or induce an immune complex type of tissue injury. The enhanced severity of respiratory syncytial virus infection in early infancy is believed to be due to the presence of passively acquired maternal antibodies. In older children who have no antibody, the virus causes a milder disease. The pathogenesis of some viral haemorrhagic fevers is believed to be immunological thrombocytopaenia. Most extrahepatic lesions of serum hepatitis are due to damage caused by immune complexes.

It has been proposed that humoral antibodies may not be important in the protection against virus infections. This view is based largely on the observation that patients with agammaglobulinaemia are able to present a normal resistance to virus infections, in contrast to their extreme susceptibility to bacterial infections. This observation may not be entirely valid, since even agammaglobulinaemic individuals produce small amounts of antibody which may be sufficient to afford protection against virus infections. The antiviral activity of humoral immunity is evident from the efficiency of maternal antibodies and of passively administered gamma globulin to prevent many virus infections.

The earliest indication of cell mediated immunity in virus infections was the demonstration of delayed hypersensitivity following vaccination in immune individuals. Similar skin reactivity is also seen in mumps. The normal resistance to virus infections shown by agammaglobulinaemics is ascribed to their cell mediated immunity. Individuals with deficient cellular immunity show a heightened susceptibility to infection by herpes, pox and measles viruses. The administration of antilymphocyte serum induces fatal infection in mice injected with a sublethal dose of ectromelia virus. Cell mediated immunity is considered to play a major role in recovery from virus infections in which viraemia is not important and in which infected cells have virus specific antigens on their surface. In some virus infections cell mediated immunity may contribute to tissue damage, as for example in lymphocytic choriomeningitis in mice.

Some viral infections cause a suppression of the immune response. Measles infection induces a temporary depression of delayed hypersensitivity to tuberculin. Infection of adult mice with lymphocytic choriomeningitis or leukaemia viruses inhibits antibody response to other antigens.

In general, virus infections are followed by solid immunity to reinfection, which may in some cases be lifelong. Apparent exceptions like the common cold and influenza are not due to lack of immunity but to reinfection being caused by antigenically different viruses. Live virus vaccines also induce more durable protection than bacterial vaccines.

Nonimmunological responses: Phagocytosis: Polymorphonuclear leucocytes do not play any significant role in the defence against virus infections. In fact, more virus diseases are characterised by a polymorphonuclear leucopaenia. On the other hand, macrophages phagocytose vir-

uses and are important in clearing viruses from the bloodstream.

Body temperature: Fever may act as a natural defence mechanism against virus infections as most viruses are inhibited by temperatures above 39°C. An exception is herpes simplex which is usually reactivated by fever to produce 'fever blisters'. Herpes febrilis is a frequent accompaniment of fevers caused by pneumococci, streptococci, influenza virus and malaria parasites but, for some unknown reason, is very rare in other fevers (smallpox, typhoid and tuberculosis).

Hormones: Corticosteriod administration enhances most virus infections. Coxsackie virus B1 does not normally cause disease in adult mice, but will induce a fatal infection in mice treated with cortisone. Normally mild infections such as varicella and vaccinia may be lethal in patients on cortisone. Injudicious use of steroids in the treatment of herpetic keratoconjunctivitis may cause blindness. The particularly severe course of many virus infections in pregnancy may be related to the hormonal changes associated with pregnancy. The deleterious effect of cortisone may be due to its depression of the immune response and inhibition of interferon synthesis.

Malnutrition: Some virus infections, such as measles, produce a much higher incidence of complications and a higher case fatality rate in malnourished children than in well fed patients.

Age: Most virus infections are commoner and more dangerous at the two extremes of age. A notable exception was the influenza pandemic of 1918–1919 which caused the highest fatality in young adults.

Interferon: Isaacs and Lindenmann (1957) observed that chick chorioallantoic membrane fragments treated with live or inactivated influenza virus produced a diffusible antiviral substance which rendered cells resistant to virus infection. They gave the name interferon to this antiviral substance. It was subsequently found that interferon production is a natural defence mechanism possessed by vertebrate cells against virus infection.

Interferons are a family of glycoproteins produced by cells on induction by viral or nonviral inducers. Interferon by itself has no direct action on viruses, but it acts on other cells of the same species, rendering them refractory to virus infection. On exposure to interferon, cells produce a protein ('translation inhibiting protein', TIP) which selectively inhibits translation of viral mRNA, without affecting cellular mRNA. It has also been suggested that inhibition of viral transcription may also be responsible for the antiviral activity of interferon.

Interferons are species specific, in that interferon produced by one species can protect only cells of the same or related species against viral infections, but not cells of unrelated species. Thus, the antiviral effect on human cells is shown by human interferon, and to some extent by monkey interferon, but not by chick or mouse interferon. The activity is not virus specific. Interferon induced by one virus (or even by nonviral inducers) can confer protection against infection by the same or unrelated viruses. However, viruses vary in their susceptibility to interferon. Viruses also vary in their capacity to induce interferon, cytocidal and virulent viruses being poor inducers and avirulent viruses being good inducers. Examples of potent inducers are togaviruses, vesicular stomatitis virus, Sendai virus and NDV. Nucleic acids (e.g., double stranded RNA) and some synthetic polymers (e.g., Poly I:C) are particularly efficient inducers. Interferon production is increased by increasing the temperature upto about 40°C and is inhibited by steroids and increased oxygen tension. Interferon synthesis begins within about an hour of induction and reaches high levels in 6–12 hours. Cellular transcription and protein synthesis are necessary for interferon production.

Based on antigenic characters, cell of origin and other properties, interferons have been classified into three types — alpha, beta and gamma. The abbreviation IFN designates interferon and species of origin is indicated as a prefix — for example, human interferon alpha is usually abbreviated as Hu IFN.

Alpha interferon (formerely known as type 1,

leucocyte or Le (IFN) is produced by B lymphocytes or macrophages on stimulation by suitable viruses. Atleast 16 antigenic subtypes have been identified.

Beta interferon (formerly known as type I, fibroblast or F IFN) is produced by fibroblasts or epithelial cells on stimulation by viruses or polynucleotides.

Gamma interferon (formerly known as type II, immune or I IFN) is produced by T lymphocytes on stimulation by antigens or mitogens.

Interferons are inactivated by proteolytic enzymes, but are unaffected by nucleases and lipases. They withstand heating at 56–60°C for 30–60 minutes and are stable over a wide range of pH (2–10), except gamma interferon which is labile at pH 2.0. They are nondialysable and not sedimented by certifugation at 100,000g for several hours. Estimates of their molecular weight have ranged from 12,000 to 100,000. This variation is probably because interferons occur in polymeric forms, the monomer having a MW of 12,000. They are virtually nonantigenic so that no serologic method is available for their detection and estimation. Interferon assay is based on its biological activity, as for example its ability to inhibit plaque formation by a sensitive virus.

Many properties of interferon make it an ideal candidate for use in the prophylaxis and treatment of viral infections; it is nontoxic, nonantigenic, diffuses freely in the body and has a wide spectrum of antiviral activity. The only drawback initially was its species specificity so that interferon produced by nonhuman cells was not clinically useful. This was overcome to some extent by producing interferon from buffy coat and leucocytes from blood banks, with NDV or Sendai virus as inducer. Now, human interferon is available in unlimited quantities following its commercial production by cloning in bacteria. But even so, its initial promise as an antiviral agent has not been fulfilled. Local application of high doses has shown some benefit against upper respiratory infections, herpetic keratitis and genital warts. Limited success has also been reported against generalised herpes infection in immunocompromised hosts. Some encouraging results have been reported in the use of interferon as an anticancer agent, particularly in lymphomas, but there have been reports of toxic effects in cancer patients given high doses of interferon.

Although interferon was first recognised as an antiviral agent, it is now known to be a more general regulatory peptide belonging to the class of *cytokines*. The main biological effects of interferons are the following:

1. Antiviral effects: Induction of resistance to infections.
2. Antimicrobial effects: Resistance to intracellular infections, e.g., toxoplasma, chlamydia, malaria.
3. Cellular effects: Inhibition of cell growth and proliferation; and of DNA and protein synthesis; increased expression of MHC antigens on cell surfaces.
4. Immunoregulatory effects: Enhanced cytotoxic activity of NK, K and T cells; activation of macrophage cytocidal activity; modulation of antibody formation; activation of suppressor T cells; suppression of DTH.

Laboratory diagnosis of viral diseases

Technical difficulties in virus isolation and identification, the length of time required for these procedures and the lack of specific therapy for virus infections have contributed to the sparse use of diagnostic virology till recently. The situation has changed in recent years. With the development of rapid techniques for the diagnosis of many virus infections and the availability of specific drugs against at least a few viruses, diagnostic virology is fast becoming a routine procedure.

The demonstration of virus infection in selected groups of persons (*screening*) is an important procedure in the prevention of some diseases (e.g., screening for HBV and HIV in blood donors). Aetiological diagnosis of virus infections is useful in many ways. It is of vital importance in some cases, as in rubella in pregnant women. It helps institution of early specific

TABLE 49.1

Types of specimens to be sent for virus diagnosis

System	Specimens required[1]. For isolation	For direct examination[2]	For serology
Respiratory	Throat swab, nasopharyngeal aspirates	Nasopharyngeal aspirate (IF) [throat washings (EM)]	Paired sera
Central Nervous System	Faeces, blood (for arbovirus isolation) CSF, (brain biopsy, throat swab, rectal swab)	Brain biopsy (IF & EM); CSF (EM & IF) (corneal impression smears IF[3]	Paired sera
Cardiovascular System	Faeces	Nil	Paired sera
Skin	Macular/papular scrapings, vesicular/pustular fluid, ulcer scrapings, crust, faeces, throat swab	Vesicular/pustular fluid (EM & ID), Ulcer scrapings (EM), crusts (EM & ID)	Paired sera
Eye	Conjunctival scrapings or swabs	Conjunctival scrapings, as smears on microscope slides (LM & IF)[3]	Paired sera
Liver	Blood (for yellow fever)	Serum (faeces)	Serum
General; congenital infections	Throat swab (products of conception)	Nil	Single sera (Mother & Baby)
General; PUO	Heparinised blood (arbovirus and arenavirus infections) throat swabs, faeces (fresh urine)	Nil	Paired sera.

1. Specimens within brackets are not appropriate for routine diagnosis but may be indicated in particular circumstances. 2. IF = immunofluorescence; EM = Electron Microscopy; ID = Immunodiffusion, LM = Light Microscopy. 3. for diagnosis of rabies only. (Adapted from WHO)

therapy as in herpetic lesions of the eye. It serves to define the aetiology of vague syndromes such as 'upper respiratory infection' or 'aseptic meningitis'. It is essential for the detection and prediction of epidemics and the identification of antigenic variation in viruses. It is invaluable in the prompt control of outbreaks. It may lead to the discovery of new virus infections.

Successful diagnosis of virus infections depends as much on the awareness of the physician as on the capability of the virus laboratory. The appropriate specimens should be collected from patients, preserved and transported to the laboratory in the proper manner along with pertinent clinical and epidemiological information (Table 49.1).

In the laboratory, the following methods are commonly employed: microscopic demonstration of the virus or its inclusion body, demonstration of the virus antigen, isolation and identification of the virus, or detection of the specific antibody.

1. Microscopy: The demonstration of virus elementary bodies by examination of stained smears is now seldom employed. The detection of virus by electron microscopy is being used increasingly. In some diseases, it used to be the only diagnostic method (e.g., viral diarrhoea). Demonstration of the inclusion body is a routine diagnostic method for rabies in dogs. The microscopic diagnosis of rabies has been rendered very sensitive by fluorescent antibody techniques. The use of direct and indirect fluorescent antibody techniques for the examination of material from lesions, as well as for the early demonstration of viral antigen in tissue cultures inoculated with specimens has enlarged the scope and greatly increased the speed of virus diagnosis.

2. Demonstration of virus antigen: In cases where virus antigen is abundant in the lesions, its demonstration by serological methods such as precipitation in gel or immunofluorescence offers a rapid method of diagnosis. Highly sensitive serological tests such as counterimmunoelectrophoresis, radioimmunoassay and enzyme linked immunosorbent assay have found wide application in diagnostic virology for the detection of viral antigens in clinical samples.

3. Isolation of virus: This is the commonest technique used in the diagnosis of virus infections. For virus isolation it is imperative that the specimen be collected properly and transported with least delay to the laboratory. As most viruses are heat labile, refrigeration is essential during transport. The methods used for isolation depend on the virus sought. In general they consist of inoculation into animals, eggs or tissue culture, after the specimen is processed to remove bacterial contaminants. The isolates are identified by neutralisation or other suitable serological procedures. It has to be emphasised that the mere recovery of a virus from a patient does not justify the assumption that it is the causative agent of the patient's illness. Many viruses (e.g., adenoviruses, enteroviruses) are frequently found in normal individuals. The results of isolation should always be interpreted in the light of the clinical data. Demonstration of an immunological response to the virus isolate in the patient during the course of the disease reinforces the significance of the isolation.

4. Serological diagnosis: The demonstration of a rise in titre of antibodies to a virus during the course of a disease is strong evidence that it is the aetiological agent. For this, it is essential to examine paired sera, the 'acute' sample collected early in the course of the disease and the 'convalescent' sample collected ten to fourteen days later. Examination of a single sample of serum for antibodies may not be meaningful. The serological techniques employed would depend on the virus, but those in general use are neutralisation, complement fixation, ELISA and haemagglutination inhibition tests.

Immunoprophylaxis of virus diseases

Prolonged and effective immunity is a charac-

teristic of most virus infections. Viral vaccines also confer solid protection and are, in general, more effective than bacterial vaccines. Viral vaccines may be live or killed (Table 49.2). Live vaccines are more effective than killed vaccines (e.g., smallpox, yellow fever). The smallpox vaccine has been used as the sole tool for the global eradication of the disease. The earlier live vaccines were developed empirically from natural viruses (e.g., Jenner's cowpox vaccine) or by attenuation by serial passage (e.g., yellow fever vaccines). The basis of the latter technique was an unconscious selection of avirulent mutants. With the development of more precise genetic techniques, live vaccines have been developed by plaque selection (e.g, Sabin vaccine for poliomyelitis) or from *ts* mutants (e.g., influenza). A more recent method has been the development of vaccine strains with the desired antigenic characters by recombination (e.g., influenza).

Killed vaccines have been prepared by inactivating viruses with heat, phenol, formalin or beta propiolactone. Ultraviolet irradiation is not satisfactory because of the risk of multiplicity reactivation. The reactogenicity of killed vaccines has been attempted to be reduced by the purification of the viruses. Adverse reactions may be reduced also by the use of 'subunit vaccines' in which the virus is split by detergents or other chemicals and only the relevant antigens incorporated in the vaccine.

Live vaccines have the following advantages: A single dose is usually sufficient. They can be administered by the route of natural infection so that local immunity is induced. They induce a wide spectrum of immunoglobulins to the whole range of viral antigens. They also induce cell mediated immunity. They provide more effective and more lasting immunity than killed vaccines. They can, in general, be prepared more economically and administered more conveniently, especially for mass immunisation. They have the following disadvantages. There is a risk, however remote, of reversion to virulence. The vaccine may be contaminated with potentially dangerous viruses such as oncogenic viruses. The virus may spread from the vaccinees to contacts. While this is a serious danger in some situations (as in rubella, if the vaccine strain is teratogenic), in other cases, it may even be an advantage (as in poliomyelitis where the range of vaccination is extended by the natural spread of the vaccine virus among children and adults). Interference by preexisting viruses may sometimes prevent a good immune response following live vaccination. Live vaccines are heat labile and they have to be kept under refrigeration. Some live vaccines may cause local and remote complications (e.g., smallpox vaccine).

Killed vaccines have the advantage of stability and safety. They can be given in combination as polyvalent vaccines. There is also no danger of the spread of the virus from the vaccinee. The disadvantages are that multiple injections are needed and that local immunity and cell mediated immunity are not induced.

Passive immunisation with human gamma globulin, convalescent serum or specific antiserum gives temporary protection against many virus diseases such as measles, mumps, and infectious hepatitis. These are indicated only when nonimmune individuals who are at special risk are exposed to infection. Combined active and passive immunisation is an established method for the prevention of rabies.

Chemoprophylaxis and chemotherapy of virus diseases

The phenomenal success achieved by antibiotics and chemotherapeutic agents in the control of bacterial diseases is in marked contrast to the virtual absence of safe and effective drugs for viral diseases. As viruses are strict intracellular parasites that use the biosynthetic mechanisms of the host cell for replication, it was feared that it may not be possible to inhibit viral replication without damaging the host cell. But it is now known that there are several areas available for attack on viruses selectively. Viral infection may be checked at the level of attachment, transcription of viral nucleic acid, translation of viral mRNA and repli-

TABLE 49.2

Viral vaccines in common use

Disease	Type of vaccine	Mode of preparation
Poliomyelitis	Live	Avirulent strains grown in monkey kidney cell culture
	Killed	Virulent strains grown in monkey kidney cell cultures, formalin-killed
Rabies	Killed (Semple type)	Fixed virus grown in sheep brain and inactivated by *phenol or beta propiolactone*
	Killed	Virus grown in cell culture and inactivated with beta propiolactone
Yellow fever	Live (17D)	Attenuated virus grown in chick embryos and lyophilised
Japanese encephalitis	Killed	Virus grown in mouse brain and inactivated by formalin
Mumps	Live	Attenuated virus grown in chick embryo fibroblast culture
Influenza	Killed (subunit)	Virus disintegrated with sodium deoxycholate
	Live (attenuated)	Virus attenuated by serial passage in eggs
	Live (mutant)	*ts* mutants which are avirulent
	Live (recombinant)	Recombinants with surface antigens of new strains and growth characters of established strains
Measles	Live	Attenuated virus grown in tissue culture
Rubella	Live	Attenuated virus grown in tissue culture
Hepatitis B	Killed	HB_sAg from human carrier sera-inactivated by HCHO

cation of viral nucleic acid. A number of virus specific enzymes have been identified which can be inhibited selectively, thereby preventing viral multiplication without affecting the host cells. Interferon is able to discriminate between viral and cellular MRNA and is a perfect model of a nontoxic antiviral drug. This opens up the logical basis for chemotherapy.

The range of antiviral chemoprophylaxis and therapy is at present very limited. Adamantanamine which inhibits the attachment of the influenza virus to cells has been found useful in prophylaxis but is much less effective in treatment. Rifampin, which inhibits viral transcription is effective against poxviruses in cell cultures. Thiosemicarbasones, which inhibit viral

translation, are of some value in the prophylaxis of smallpox, but are ineffective therapeutically. Iododeoxyuridine which inhibits replication of viral nucleic acid is too toxic for systemic administration but is effective in the topical therapy of herpetic keratoconjunctivitis. It has also been used as a life saving measure in herpes encephalitis. Trifluoromethyl deoxy uridine and cytosine arabinoside have also been tried in the treatment of herpes virus infection. The purine analogue adenosine arabinoside (Vidarabine, Ara-A) is relatively nontoxic and is clinically effective in herpesvirus infections. Ribavirin, a synthetic nucleoside, inhibits the multiplication of many DNA and RNA viruses, *in vitro* and in experimental animals. Ribavirin has been found to be effective against influenza and RSV infections when administered as an aerosol.

Acyclovir (acycloguanosine) which is soluble, stable and highly potent has emerged as a very useful drug in the treatment of herpetic encephalitis and superficial lesions. It may be administered locally, orally or IV. It is effective against herpes simplex and varicella-zoster but not against cytomegalovirus. Its activity depends on viral thymidine kinase and is therefore nontoxic to host tissues.

Zidovudine (azidothymidine) is of some value against HIV and has been licensed for use in the treatment of AIDS.

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50 Bacteriophage

Bacteriophages (commonly abbreviated as phages) are viruses that infect bacteria. Twort (1915) described a degenerative change in staphylococcal colonies isolated from calf lymph, which could be transmitted serially by application of culture filtrates from the original growth. d'Herelle (1917) observed that the filtrates of faeces cultures from dysentery patients induced transmissible lysis of a broth culture of a dysentery bacillus. He suggested that the lytic agent was a virus and gave it the name bacteriophage.

Phages occur widely in nature in close association with bacteria. They can be readily isolated from faeces, sewage and other natural sources of mixed bacterial growth. Early hopes that phages could be used in the treatment of bacterial infections have not been fulfilled, but these viruses have contributed much to microbiology. As phages could be grown easily on bacterial cultures, they provided the only convenient model for the study of virus-host interactions at the cellular and molecular levels before the development of cell culture techniques made similar studies with animal viruses possible. Phages play an important role in the transmission of genetic information between bacteria by the process of transduction. The presence of phage genome integrated with bacterial chromosomes confers on bacteria certain properties by the process known as phage conversion. The specificity of the host range of phages is the basis of phage typing methods, by which bacteria can be identified and typed.

Morphology

Certain bacteriophages that infect *E. coli*, called the T even phages (T2, T4, T6), have been studied in great detail and traditionally serve as the prototypes in describing the properties of bacteriophages.

T even phages have a complex and characteristic morphology. They are tadpole shaped, with a hexagonal head and a cylindrical tail. The head consists of a tightly packed core of nucleic acid (double stranded DNA) surrounded by a protein coat or capsid. The size of the head varies in different phages from 28 nm to 100 nm. The tail is composed of a hollow core, a contractile sheath surrounding the core and a terminal base plate which has attached to it prongs, tail fibres or both (Fig. 50.1)

Though most bacteriophages have the morphology and structure described above, phages that are spherical or filamentous and possess single stranded DNA or RNA have been identified.

Life cycle

Phages exhibit two different types of life cycle. In the *virulent* or *lytic* cycle, intracellular multiplication of the phage culminates in the lysis of the host bacterium and the release of progeny virions. In the *temperate* or *lysogenic* cycle the phage DNA becomes integrated with the bacterial genome, replicating synchronously with it, causing no harm to the host cell (Fig. 50.2).

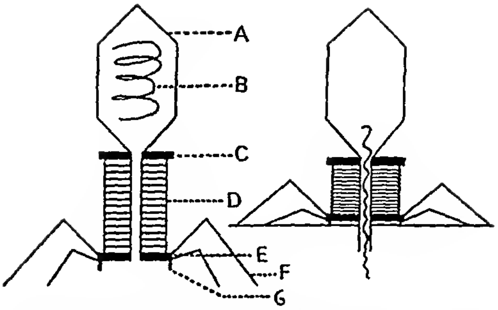

Fig 50.1 Morphology of bacteriophage. A. hexagonal head, B DNA core, C. demarcation between head and tail, D. tail, E base plate, F. tail fibres, G prongs. Right. Process of injection of phage DNA into host cell

Lytic cycle: Replication of a virulent phage can be considered in the following stages — adsorption, penetration, synthesis of phage components, assembly, maturation and release of progeny phage particles.

Phage particles come into contact with bacterial cells by random collision. A phage attaches to the surface of a susceptible bacterium by its tail. Adsorption is a specific process and depends on the presence of complementary chemical groups on the receptor sites of the bacterial surface and on the terminal base plate of the phage. Under optimal conditions, adsorption is a very rapid process, being complete within minutes. Certain cofactors, such as cations, are necessary for adsorption. The bacterial receptor sites may be situated in different layers of the cell wall or on surface structures (such as the Vi antigen of the typhoid bacillus) or appendages (such as flagella or sex pili). Bacterial protoplasts, which are devoid of cell wall components cannot adsorb phage and, therefore, will not be infected. Host specificity of phages is determined at the level of adsorption. Experimental infection by direct injection of phage DNA can be achieved even in bacterial strains that are insusceptible to infection by the whole phage. The infection of a bacterium by the naked phage nucleic acid is known as *transfection*.

Adsorption is followed by the penetration of the phage nucleic acid into the bacterial cell. The process of penetration resembles injection through a syringe. The base plate and tail fibres are held firmly against the cell causing the hollow core to pierce through the cell wall. The contractile tail sheath acts like a muscle and derives its energy from a small amount of adenosine triphosphate present on the tail of the phage. The phage DNA is injected into the bacterial body through the hollow core. Penetration may be facilitated by the presence on the phage tail of lysozyme which produces a hole on the bacterial wall for the entry of the phage core. The complex structure of the phage particle is required only for the injection of the nucleic acid into the host cell. The phage DNA alone is necessary for the initiation of the

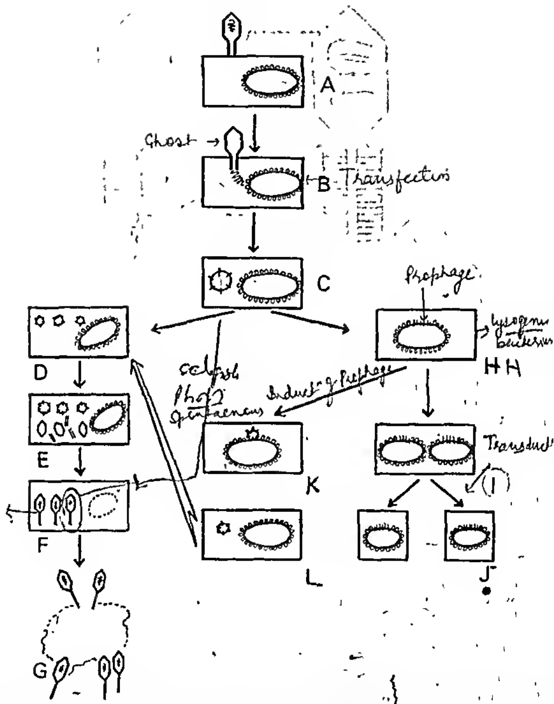

Fig. 50.2 Lytic and lysogenic life of bacteriophage. A adsorption B injection of phage DNA, C circularisation of phage DNA, D replication of phage DNA, E production of phage components, F assembly of phage, G release of progeny phage, H integration of phage DNA with host chromosome, I binary fission of lysogenic bacterium, J daughter bacteria carrying prophage, K excision of prophage, L, same stage as C (A to C infection D to G lytic cycle; H to J lysogenic cycle; K to L induction)

eclipse ... of phage intracellularly
latent period → Interval betn infectn of bact to release of infectious phage particle.

synthesis of daughter phages. After penetration, the empty head and tail of the phage remain outside the bacterium as the shell or '*ghost*'.

When bacteria are mixed with phage particles at high multiplicity (i.e., very large number of phages per bacterial cell), multiple holes are produced on the cell with the consequent leakage of cell contents. Bacterial lysis occurs without viral multiplication. This is known as 'lysis from without'.

Immediately after penetration of the phage *nucleic acid*, the synthesis of the phage components is initiated. The first products to be synthesised (called *early proteins*) are the enzymes necessary for the building of the complex molecules peculiar to the phage. Subsequently, *late proteins* appear, which include the protein subunits of the phage head and tail. During this period, the synthesis of bacterial protein, DNA and RNA ceases.

Phage DNA, head protein and tail protein are synthesised separately in the bacterial cell. The DNA is condensed into a *compact polyhedron* and 'packaged' into the head and, finally, the tail structures are added. This assembly of the phage components into the mature infective phage particle is known as maturation.

Release of the *mature progeny phages* typically occurs by lysis of the bacterial cell. During the replication of phage, the bacterial cell wall is weakened and it assumes a spherical shape. *Phage enzymes* act on the weakened cell wall causing it to *burst or lyse* resulting in the release of mature daughter phages.

The interval between the entry of the phage nucleic acid into the bacterial cell and the appearance *of the first* infectious intracellular phage particle is known as the *eclipse phase*. It represents the time required for the synthesis of the phage components and their assembly into mature phage particles. The interval between the infection of a bacterial cell and the first release of infectious phage particles is known as the *latent period*. Immediately following the latent period the number of phage particles released increases for a few minutes till the maximum number of daughter phages is attained. This period, during which the number of *infectious* phages released rises, is known as the *rise period*. The average yield of progeny phage per infected bacterial cell is known as the *burst size*. This is estimated by *experiments* in which infection is established with one phage per bacterium and *the release of* infected phage particles is estimated serially over a period of time. The results of such an experiment plotted on a graph is known as the one-step growth curve (Fig. 50.3).

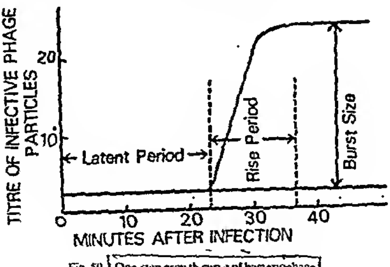

Fig. 50 One-step growth curve of bacteriophage

Lysogenic cycle: Unlike virulent phages which produce lysis of the host cell, temperate phages enter into a symbiotic relationship with their host cells without destroying them. Following entry into the host cell, the temperate phage nucleic acid becomes integrated with the bacterial chromosome. The integrated phage nucleic acid is known as the *prophage*. The prophage behaves like a segment of the host chromosome and replicates synchronously with it. This phenomenon is called *lysogeny* and a bacterium that carries a prophage within its genome is called a lysogenic bacterium. Lysogenisation does not upset the bacterial metabolism.

The prophage confers certain new properties on the lysogenic bacterium. This is known as *lysogenic conversion* or *phage conversion*. This is due to the synthesis of new proteins that are coded for by the prophage DNA. An example is toxin production by the diphtheria bacillus, which is determined by the presence in it of the prophage *beta*. The elimination of the prophage abolishes the toxigenicity of the bacillus.

During the multiplication of lysogenic bacteria the prophage may become 'excised' from occasional cells. The excised prophage initiates lytic replication and the daughter phage particles are released, which infect other bacterial cells and render them lysogenic. This is known as 'spontaneous induction of prophage'. While this is a rare event, all lysogenic bacteria in a population can be induced to shift to the lytic cycle by exposure to certain physical and chemical agents. Such inducing agents include UV rays, hydrogen peroxide and nitrogen mustard.

A lysogenic bacterium is resistant to reinfection by the same or related phages. This is known as *superinfection immunity*.

Bacteriophages may act as carriers of genes from one bacterium to another. This is known as *transduction*. Two types of transduction are recognised. In restricted transduction, only bacterial genes contiguous to the prophage are transmitted. For example, transduction by the prophage *lambda* in *E. coli* K 12 transfers only the gal+ gene (determining fermentation of galactose), which is the bacterial gene contiguous to the prophage. On the other hand, any bacterial gene may be transferred in generalised transduction. Transduction has been demonstrated in many genera of bacteria and constitutes one of the most important mechanisms of genetic exchange among bacteria in nature. Plasmid mediated drug resistance in staphylococci is an example of a medically important property that is transmitted by transduction.

Phage particles exhibit general stability of type and a low rate of heritable variation.

If a bacterium simultaneously adsorbs two related but slightly different DNA phage particles, both can infect and reproduce. On lysis, both types are released. When this occurs many of the progeny are observed to be recombinants.

Phage assay

When a phage is applied on the lawn culture of a susceptible bacterium, areas of clearing occur after incubation. These zones of lysis are called 'plaques'. The size, shape and nature of plaques are characteristic for different phages. Since under optimum conditions a single phage particle is capable of producing one plaque, plaque assay can be employed for titrating the number of viable phages in a preparation. As plaques are analogous to bacterial colonies, plaquing is also useful for the purification of phages.

Phage typing

The specificity of phage-bacterium interaction is made use of in the methods for the identification and typing of bacteria. Phages exhibit different degrees of host specificity. Some phages possess wide host ranges, covering many bacterial genera, while others have a narrow range limited to certain strains of bacteria only. With some phages serial passage in a strain of bacterium makes them specific for that strain and related strains (adaptation of host range).

Phages are available that lyse all members of a bacterial genus (e.g., genus specific bacteriophage

for *Salmonella*), all members of a species (e.g., specific bacteriophage for *B. anthracis*), and all members of a biotype or subspecies (e.g., Mukerjee's phage IV which lyses all strains of classical *V. cholerae*, but not *V. cholerae* biotype el Tor). The most important application of phage typing is for intraspecies typing of bacteria, as in the phage typing of *S. typhi* and staphylococci. Adapted phages, active only against fresh isolates possessing the Vi antigen, are used for phage typing of typhoid bacilli. Staphylococcal phage typing is a pattern method, using a set of standard phages. A strain of staphylococcus may be lysed by a number of phages and the phage type of a strain is designated by the numbers of the different phages that lyse it.

As lysis is influenced by the dose of infection, phage preparations used for typing should be standardised by titration. Titration is carried out by applying serial dilutions of the phage preparation on a lawn culture of a susceptible strain and observing the lysis after incubation. The highest dilution of the phage preparation that just produces confluent lysis is known as the 'routine test dose' (RTD).

5 **Bacteriocins** proteins (mainly), + Lipopolysaccharides

Gratia (1925) observed the production of a highly specific antibiotic substance by one strain of *E. coli* which was active against another strain of the same species. The name colicin was given to such substances produced by *E. coli* and other members of the family Enterobacteriaceae. With the recognition that colicin-like substances are produced by several other bacteria also, the generic name bacteriocin was proposed for the group of highly specific antibiotic-like substances produced by certain strains of bacteria and active against other strains of the same or different species. Bacteriocins are given specific names based on the bacterial species of origin, e.g., colicins from *E. coli*, pyocins from *Ps. pyocyanea (aeruginosa)*, megacins from *B. megaterium* and diphthericins from *C. diphtheriae*.

Bacteriocins are proteins, but some may have

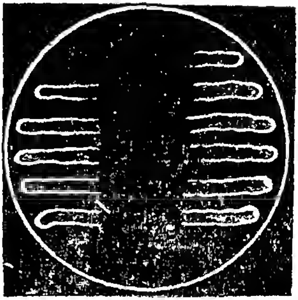

Fig 50.4 Bacteriocin typing. Bacteriocin produced by the producer strain has inhibited the growth of test strains in the centre.

associated lipopolysaccharides derived from the cell walls of bacteria producing them. Bacteriocins and phages resemble each other in a number of respects. 1 Both adsorb on the surface of susceptible bacterial cells on specific receptor sites some of which may be the same for phages and bacteriocins. 2 Under the electron microscope, some bacteriocins, especially pyocins appear like the tail structures of phages. They may be considered to be products of defective phage genomes, able to code only for parts of phage particles.

The synthesis of bacteriocins is determined by the presence in bacteria of colicinogenic factors (Col factors). Col factors are episomes and can be transmitted from cell to cell by conjugation or transduction. Certain physical and chemical agents (UV rays, nitrogen mustard) induce colicin production by the cells harbouring Col factors.

A cell producing a bacteriocin is immune to it, but may be sensitive to other bacteriocins. Bacteriocins have a very specific activity on bacteria, being capable of killing some but not all strains of

a species. The specificity is made use of in typing certain species such as *Sh. sonnei*, *Proteus sp.*, *Ps. aeruginosa*. Bacteriocins kill susceptible cells without lysing them.

While phage typing schemes are generally based on the sensitivity of the test strains to the lytic action of phages, bacteriocin typing schemes depend on the ability of bacteriocins produced by the test strain to kill standard indicator strains of bacteria. The usual method of bacteriocin typing employs the plate diffusion technique. The test bacterium is inoculated as a broad streak on the centre of a culture medium, the bacterial growth is scraped off and the remaining cells killed by exposure to chloroform vapour. Standard indicator strains of bacteria are then streaked at right angles to the original inoculum. After incubation, the pattern of inhibition of the indicator strains represents the bacteriocin type of the test bacterium (Fig 50.4).

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51 Poxviruses

Poxviruses are the largest viruses that infect vertebrates, large enough to be seen under the light microscope. This group contains several viruses that infect man, animals, birds and insects. Based on genetic, antigenic and ecological criteria, the Family Poxviridae has been classified into two sub-families: *Chordopoxvirinae*, the poxviruses of vertebrates, and *Entomopoxvirinae*, the poxviruses of insects which do not infect vertebrates.

Chordopoxivirinae are placed in six genera or subgroups:

1. *Orthopoxvirus*: Mammalian poxviruses that tend to cause generalised infection with rash — variola, vaccinia, cowpox, monkeypox, rabbitpox, buffalopox, camelpox, mousepox.
2. *Parapoxvirus*: Viruses of ungulates that may occasionally infect man, orf (contagious pustular dermatitis), paravaccinia (milker's nodes, bovine papular stomatitis).
3. *Capripoxvirus*: Viruses of goats and sheep — sheeppox, goatpox, lumpyskin disease.
4. *Leporipox virus*: Viruses of leporids (rabbits, hares, squirrels) — myxoma and fibromas.
5. *Avipoxvirus*: Viruses of birds — fowlpox, turkeypox, pigeonpox, canarypox.
6. *Suipoxvirus*: Swinepox.

Poxviruses that have not been officially assigned to any genus include the virus of molluscum contagiosum, tanapox and the yaba monkey tumour.

Poxvirus diseases are characterised by skin lesions which may be localised or generalised. The most important of these was smallpox caused by the variola virus. Other poxviruses which can infect man are vaccinia, cowpox, monkeypox, tanapox, molluscum contagiosum, paravaccinia and orf. Buffalopox and camelpox may occasionally infect man causing lesions resembling vaccination.

Variola and vaccinia: The variola virus is the causative agent of smallpox. For thousands of years, smallpox raged as a scourge of mankind causing death and disfigurement. The global eradication of smallpox, achieved after 10 years of concerted campaigns under the auspices of the WHO, has been the most impressive medical achievement. Naturally occurring smallpox appears to have come to an end in 1977. On 8 May, 1980, the WHO formally announced the global eradication of smallpox.

Smallpox used to occur in two distinct clinical varieties — the florid, highly fatal disease typically seen in Asia, and the mild, nonfatal disease (alastrim) typically seen in Latin America. The virus causing classical smallpox was called *variola major* and that causing alastrim *variola minor*. Variola major and minor are antigenically identical, but they differ in certain biological characteristics. They seem to be stable variants as the disease produced by each always breeds true; alastrim did not lead to smallpox and *vice versa*.

The vaccinia virus is used as the smallpox vaccine. Jenner originally used the cowpox virus for vaccination against smallpox but during the several years in which the original vaccine virus was maintained by arm to arm passage in man, it underwent some permanent changes so that it can now be readily differentiated from the fresh isolates of the cowpox virus. The vaccinia virus is unique in that it is an 'artificial virus' and does not

occur in nature as such. It has been studied in greater detail than variola, as it is safer to work with. Vaccinia and variola viruses are so similar in their properties that they can be considered together.

Morphology: The virion is brick shaped. In vertical section, it consists of a double layered membrane which surrounds a biconcave 'nucleoid' containing the DNA core. On either side of the nucleoid is a lens shaped structure called the lateral body (Fig. 51.1). The virion measures about 300 x 200 x 100 nm and so can be seen under the light microscope. Variola virus was first demonstrated microscopically by Buist in 1887. Paschen in 1906 developed a staining technique for the virus particles and demonstrated the elementary bodies (Paschen bodies) in smears from small pox lesions.

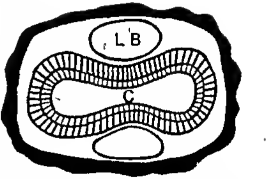

Fig 51.1 Structure of vaccinia virus. The nucleic acid is contained within a dumbell-shaped core (C). Filling into the concavities of the core are two lateral bodies (LB). The virion is enclosed within a protein shell which has an irregular surface

Physical and chemical properties: Poxviruses are stable and if protected from sunlight may remain viable for months at room temperature. In the cold or when freeze dried, they survive for years. They are susceptible to ultraviolet light and other irradiations. They are resistant to 50% glycerol and 1% phenol, but are readily inactivated by formalin and oxidising disinfectants. The virion consists essentially of DNA, protein and lipid. Though enveloped, the virus is not inactivated by ether. The virion contains a multiplicity of enzymes. The entire multiplication of the virus takes place in the cytoplasm of the infected cell.

Antigenic structure: All poxviruses share a common nucleoprotein (NP) antigen. By immunodiffusion some twenty different antigens have been identified. These include the LS antigen (a complex of two antigens, the heat labile L and the heat stable S antigens), agglutinogen, and haemagglutinin, which is responsible for the agglutination of erythrocytes of those fowls which are also agglutinated nonspecifically by tissue lipids

Cultivation and host range: The variola and vaccinia viruses can be differentiated by their growth characteristics and host range.

Chick embryo: Both viruses grow on the CAM of 11 – 13 day old chick embryos producing pocks in 48 – 72 hours. Variola pocks are small, shiny, white, convex, nonnecrotic, nonhaemorrhagic lesions. Vaccinia pocks are larger, irregular, flat, greyish, necrotic lesions, some of which are haemorrhagic (Fig. 51.2). The viruses may also be differentiated by their 'ceiling temperatures', the highest temperature above which pocks are not produced. The ceiling temperatures are 41.0°C for vaccinia, 38°C for variola major and 37.5°C for variola minor.

Tissue culture: The variola and vaccinia viruses can be grown in tissue cultures of monkey kidney, HeLa and chick embryo cells. Cytopathic effects are produced by vaccinia in 24 – 48 hours and by variola more slowly. Eosinophilic inclusion bodies, Guarnieri bodies, can be demonstrated in stained preparations. The inclusion bodies consist of aggregations of virus particles in a matrix. Vaccinia but not variola virus produces plaques in chick embryo tissue cultures.

Animals: The vaccinia virus can infect a wide range of animals experimentally. Monkeys, calves, sheep and rabbits can be infected by scarification leading to vesicular lesions. The var-

cytopathic effect →
Vac. – 24-48 hrs. Var. – slowly

TABLE 51.1
Comparison of properties of some Orthopoxviruses

	Variola	Whitepox	Monkeypox	Vaccinia	Cowpox	Camelpox
Isolated from	Man	Ape, monkey rodent	Man, monkey anteater	Origin unknown	Man, cow, large felines	Camel
Pocks on CAM	Small, white	Small, white	Small, pink	Large, white to grey	Haemorrhagic	Small, white
Ceiling temperature on CAM (°C)	37.5–38.5	38.5	39	41	39.5	38.5
Growth on rabbit skin	–	–	++	+ or ++	++	+
Pathogenicity for baby mice	Low	Low	High	High	High	Low
Antigens specific for: Vaccinia	–	–	–	+	+	+
Antigens specific for: Variola	+	+	–	+	?	+
Antigens specific for: Monkeypox	–	–	+	–	?	–
Polypeptide pattern	Character of variola	Character of variola	Character of monkeypox	Character of vaccinia	Character of cowpox	?
Thymidine kinase sensitivity	+	+	–	–	–	–

Fig 51 2 Variola and vaccinia pocks on CAM Left—variola, showing small, uniform pocks. Right—vaccinia, showing large, irregular pocks

iola virus produces similar lesions only in monkeys. Scarification of rabbit cornea with variola virus leads to keratitis and sections of the cornea will show typical Guarnieri bodies. Intranasal instillation of the variola virus in the monkey produces a self-limited attack of smallpox with generalised skin lesions.

SMALLPOX

Smallpox has been eradicated, the last natural case having occurred in Merca, Somalia, in October, 1977 It is unlikely now that we would see any case of smallpox

But the last case of smallpox, however, occurred in August, 1980, in Birmingham, England, during a small outbreak caused by accidental infection from the Microbiology Department of the Medical School where work on variola virus was being conducted. Realising the potential danger of variola virus stocks being held in laboratories, all such stocks have been destroyed At present, the virus is stored only in two authorised laboratories, the Centre for Disease Control, Atlanta, U S A , and the Institute for Virus Preparations, Moscow, under maximum security precautions

(Those interested in further details about smallpox are referred to the earlier edition of this book)

OTHER POXVIRUS DISEASES

With the elimination of smallpox, it has become important to identify and characterise other orthopoxviruses which can infect man and cause disease resembling smallpox (Table 51.1).

Monkeypox: This virus was first isolated in 1958 from an outbreak of pox disease in a captive monkey colony in Copenhagen Similar outbreaks have since been identified in other monkey colonies also. No simian outbreaks in nature have been recorded. The first human case was reported from Zaire in 1970 Several cases have been reported from Central and West Africa

The cases clinically resembled smallpox. But person to person transmission appears to be rare. Serological studies have shown evidence of widespread natural infection in monkeys in Africa. The virus can be distinguished from variola.

Whitepox: This virus was isolated in 1964 from kidney cultures of cynomolgus monkeys imported from Malaysia. It has since been isolated from chimpanzees, monkeys and rodents from Zaire. There has been no instance of human infection. But, in laboratory tests, the virus is indistinguishable from the variola virus. It has

been suggested that whitepox may be only the laboratory pickups of smallpox virus.

Lenny virus: This was isolated in 1969 from a person with severe vesicular disease resembling smallpox in Nigeria. Laboratory studies suggested that this could be a hybrid of variola and vaccinia viruses. No similar episode has been reported afterwards.

Cowpox and milker's nodes

Both these infections are obtained from cows. Cowpox lesions are seen on the udder and teats of cows and may be transmitted to man during milking. The lesions in man usually appear on the hands or fingers and resemble primary vaccinia The disease is associated with some fever and constitutional symptoms. Cowpox virus resembles variola and vaccinia antigenically, but can be differentiated by the haemorrhagic lesions it produces on CAM and rabbit skin.

Cowpox infection has been observed only in Britain and Europe. There have been outbreaks of fatal cowpox infection in wild animals kept in zoos, including cheetahs and elephants. Natural infection has been observed in domestic cats. It has been suggested that the primary host of cowpox may not be cows, but more likely wild rodents.

Milker's node (*paravaccinia*) is a trivial occupational disease that man gets by milking infected *cows. The lesions are small ulcerating nodules* The virus is unrelated to cowpox and does not grow in eggs. It can be grown in bovine kidney cultures. It resembles *orf* virus morphologically.

Orf (contagious pustular dermatitis)

Orf is a disease of sheep and goats transmitted to man by contact. In man, the disease occurs as a single papulovesicular lesion with a central ulcer, usually on the hand, forearm or face. The virus s unrelated to the variola-vaccinia group and resembles paravaccinia virus morphologically.

Tanapox

This virus was isolated from epidemics of a febrile illness along the Tana river in Kenya in 1957–62 The patients had a single pock-like lesion on the upper part of the body. The virus is antigenically unrelated to other poxviruses and does not grow in eggs. It can be grown in human and monkey tissue cultures. Monkeys are the only animals susceptible. The virus is now active in Africa, particularly in Zaire. A similar virus has been isolated from outbreaks of disease in primate colonies in America.

Molluscum contagiosum

This disease, seen usually in children and young adults, is characterised by pink or pearly white wart like nodules on the skin. Sections of the lesions show large (20–30μ) eosinophilic hyaline inclusion bodies which displace the nuclei to the margins. These molluscum bodies are composed of large numbers of virus particles, embedded in a protein matrix. Man in the only susceptible host and the virus cannot be grown in eggs, tissue cultures or animals.

The incidence of molluscum contagiosum as a sexually transmitted disease in young adults is increasing. When it ocurs in the genital areas, it may become inflamed and ulcerated and may simulate HSV infections. Sometimes electron microscopy may be needed to demonstrate the virus in the lesions.

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52 Herpesviruses

This group consists of a number of DNA viruses that affect man and animals, characterised by the ability of establishing latent infections. The virus particle is about 100–200 nm in diameter, consisting of an icosahedral capsid with 162 capsomers and surrounded by a lipid envelope (Fig. 52.1). It is heat labile and sensitive to ether and bile salts. Herpesviruses multiply in the nuclei of infected cells and produce Cowdry type A intracellular inclusions (Lipschutz inclusions).

The family *Herpesviridae* is divided into three subfamilies based on biological, physical and genetic properties:

Alphaherpesvirinae, with a relatively short replicative cycle (12–18 hours), a variable host range and a tendency to cause latent infection in ganglia. In culture they are rapidly cytopathic and infectious virus may be released from cells, e.g., herpes simplex virus, varicella-zoster virus.

Betaherpesvirinae, which replicate slowly (more than 24 hours), have a narrow host range, grow best in fibroblasts with a tendency to produce enlargement of infected cells (cytomegaly and cause latent infection of salivary gland and other organs. *In culture,* cytopathic effect is slow and the virus remains cell associated, e.g., cytomegalovirus.

Gammaherpesvirinae, which have a narrow host range, replicate in lymphoblastoid cells, specific for either B or T lymphocytes and frequently cause latent infection in lymphoid tissue, e.g., Epstein-Barr virus.

Herpesviruses of medical importance are herpes simplex (Herpes hominis) types 1 and 2; B virus (Herpesvirus simiae); varicella-zoster; cytomegalovirus; and EB virus.

HERPES SIMPLEX

Herpes simplex occurs naturally only in man, but the virus can produce experimental infection in many laboratory animals. There are two types of herpes simplex virus. Type 1 is usually isolated from lesions in and around the mouth and is transmitted by direct contact or droplet spread from cases or carriers. Type 2 is responsible for the majority of genital tract infections and is transmitted venereally. Intracerebral inoculation in rabbits and mice leads to encephalitis, and corneal scarification produces keratoconjunctivitis in rabbits. The virus grows in a variety of primary and continuous cell cultures (monkey or rabbit kidney, human amnion, HeLa) producing cytopathic changes, well defined foci with heaped up cells and syncytial or giant cell formation. On chick embryo CAM, small (diameter less than 0.5 mm) white shiny nonnecrotic pocks are produced (Fig. 52.2). The two types of virus cross react serologically. They can be differentiated by the following features:

1) Antigenic differences can be made out using type specific sera ideally with monoclonal antibodies. 2) On chick embryo CAM type 2 strains form larger pocks resembling variola. 3) Type 2 strains replicate well in chick embryo fibroblast cells, while type 1 strains do so poorly. 4) The infectivity of type 2 is more temperature

Fig. 52.1 Herpes simplex virus. A single oval virus from intranuclear inclusion in a liver cell in a child with herpes virus infection x 100,000 (Courtesy Prof. Arun R. Chitale Jaslok Hospital, Bombay)

sensitive than that of type 1. 5) Type 2 strains are more neurovirulent in laboratory animals than type 1. 6) Type 2 strains are more resistant to antiviral agents like IUDR and cytarabine in culture. 7) Restriction endonuclease analysis of viral DNA enables differentiation between the two types as well as between strains within the same type.

Pathogenesis : Herpes simplex is one of the commonest virus infections of man, about 60–80 per cent of adults showing serological evidence of infection. Primary infection is usually acquired in early childhood, between two and five years of age. Man is the only natural host and the sources of infection are saliva, skin lesions or respiratory secretions from cases of herpetic pharyngitis. Asymptomatic carriers form the more important source of infection, especially in genital infection with type 2 strains. Transmission occurs by close contact and may be venereal in genital herpes.

After contact, the virus probably enters the tissues through defects in the skin or mucous membranes. There is then a phase of local multiplication with cell to cell spread and the local lymph nodes are involved. The virus enters cutaneous nerve fibres and is transported intra-axonally to the ganglia where it replicates. Centrifugal migration of the virus can take place from the ganglia to the skin and mucosa to cause cutaneous and mucosal lesions. The virus remains latent in the ganglia, particularly of the trigeminal and sacral nerves, to be reactivated periodically in some individuals causing recurrent oral and genital lesions. Reactivation can occur in the presence of humoral antibodies. CMI is more important in resistance to and recovery from herpes simplex infections.

The typical herpes lesions are thin walled umbilicated vesicles that rapidly become pustules and scabs, and heal without scarring. In general, primary infections, though self-limited, are more severe and widespread and associated with systemic manifestations. Recurrent infections are more localised.

Clinical features

The clinical manifestations and course depend on the site of infection, age and immune status of the host, and the antigenic type of the virus.

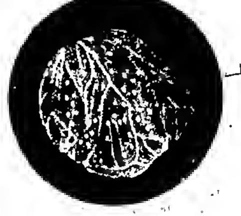

Fig. 52.2 Herpesvirus pocks on CAM. Tiny, white, uniform pocks

Cutaneous infections: The commonest site is the face — on the cheeks, chin, around the mouth or on the forehead. Lesions may also appear on the buttocks in infants as napkin rash.

The typical lesion is the fever blister or herpes febrilis, caused by viral reactivation in febrile patients. In some sensitive persons, very minor stimuli, like common cold, exposure to sun or even mental strain or menses, may bring on such reactivation.

An occupational variety of cutaneous herpes is the 'herpetic whitlow' seen in doctors, dentists and nurses.

Eczema herpeticum is a generalised eruption caused by herpes infection in children suffering from eczema. Crops of vesicles appear on the affected area with widespread ulceration. A clinically indistinguishable picture is also produced by vaccinia virus infection, both designated *Kaposi's varicelliform eruption.*

Mucosal: The buccal mucosa is the site most commonly affected. Gingivostomatitis and pharyngitis are the most frequent conditions in primary infection and recurrent herpes labialis in recurrent infection. The vesicles may ulcerate and become secondarily infected. An association has been proposed between HSV infection and oral cancer.

Ophthalmic: HSV infection is the most common cause of corneal blindness in the USA. Acute keratoconjunctivitis may occur by itself or by extension from facial herpes. Follicular conjunctivitis with vesicle formation on the lids is another manifestation. The cornea may be involved, with typical branching dendritic ulcers. Debridement, topical antiviral drugs and interferon help in healing. Steroids are contraindicated as they lead to deep stromal involvement and healing may be delayed, with scarring and corneal blindness. Superficial ulcers heal in 2–3 months even without treatment. Chorioretinitis and acute necrotising retinitis are uncommon but serious manifestations.

Nervous system: HSV encephalitis is the most common viral infection of the CNS in the USA. It may result from primary infection, the virus spreading by way of the olfactory bulb in children and young adults. In older persons, it may be due to reinfection or reactivation. HSV encephalitis has an acute onset, with fever and focal neurologic, especially temporal lobe, symptoms. Brain biopsy helps in diagnosis and instituting early specific therapy. Intravenous acyclovir reduces mortality and morbidity, but neurologic sequelae are frequent.

HSV meningitis is a self-limiting disease, usually resolving in about a week, without sequelae. CSF shows lymphocytic pleocytosis and yields the virus in culture.

HSV can cause sacral autonomic dysfunction and rarely also transverse myelitis or Guillain-Barre syndrome.

Visceral: HSV oesophagitis may cause dysphagia, substernal pain and weight loss. It may involve the respiratory tract causing tracheobronchitis and pneumonitis. HSV is an uncommon cause of hepatitis. Erythema multiforme may be seen in association with HSV infection. Disseminated HSV infection may occur in patients with immunodeficiency, malnutrition or burns.

Genital: In the 1970's, genital herpes became the most rapidly increasing venereal disease, particularly in the USA. In the male, the lesions occur *mainly on the penis, or in the urethra causing urethritis.* In the female, the cervix, vagina, vulva and perineum are affected. When only the cervix is involved, the infection may be asymptomatic. The primary infection is followed by several recurrent episodes. The vesiculo-ulcerative lesions may be very painful. Rectal and perianal lesions occur in homosexuals. Both types of HSV may cause genital lesions, though HSV-2 is responsible far more frequently and cause many more recurrences.

There have been several reports of an association between HSV-2 and carcinoma of the cervix uteri, but a causal relationship has not been established.

Congenital: Transplacental infection of the fetus with HSV-1 or 2 may lead to congenital malformations. Infection may occur during birth from lesions in the birth canal. The infection may be subclinical or localised to the skin, mouth or eyes. Generalised infection may cause death or brain damage in the survivors. Neonatal infection may also occur following postnatal exposure to the virus.

Laboratory diagnosis: The diagnosis of herpesvirus infection may be made by microscopy, virus isolation or by serology.

Microscopy: The Tzanck smear is a rapid, sensitive and inexpensive diagnostic method. Smears are prepared from the lesions, preferably from the base of vesicles and stained with 1% aqueous solution of toluidine blue 0 for 15 seconds. Multinucleated giant cells with faceted nuclei and homogeneously stained 'ground glass' chromatin (Tzanck cells) constitute a positive smear. Intranuclear Type A inclusion bodies may be seen in Giemsa stained smears. The virus particle may also be demonstrated under the electron microscope. It is not possible to differentiate between herpes simplex and varicella-zoster by microscopy. Herpesvirus antigen may be demonstrated in smears or sections from lesions by the fluorescent antibody technique.

Virus isolation: Inoculation intracerebrally in mice and on chick embryo CAM is seldom used now as they are relatively insensitive. Tissue culture is the method of choice for isolation. Primary human embryonic kidney, human amnion and rabbit kidney cells or diploid human lung and foreskin fibroblasts are inoculated with vesicle fluid. Spinal fluid, saliva and other specimens may also be used. Typical cytopathic changes may appear as early as in 24–48 hours, but cultures should be observed for two weeks before being declared negative.

Differentiation between HSV types 1 and 2 may be made by a variety of serological techniques or by nucleic acid hybridisation or by restriction endonuclease cleavage and electrophoretic analysis of viral DNA or viral proteins.

Serology: Serological methods are useful in the diagnosis of primary infections only. Antibodies develop within a few days of infection and may be demonstrated by neutralisation or complement fixation tests. In recurrent or reinfection herpes, there is little change in the antibody titre.

Chemotherapy: Ocular lesions respond to topical application of iodoxyuridine (5-iodo-2'-deoxyuridine), trifluorothymidine, vidarabine (adenine arabinoside, ara-A), acyclovir (acycloguanosine) and other inhibitors of viral DNA synthesis. Acyclovir and Vidarabine may help in herpes encephalitis if treatment is started early. Acyclovir is beneficial in primary genital herpes, but not in recurrences. Type 2 infection is more resistant to treatment than type 1.

HERPESVIRUS SIMIAE (B VIRUS)

This virus was isolated by Sabin and Wright (1934) from the brain of a laboratory worker who developed fatal ascending myelitis after being bitten by an apparently healthy monkey. It came to be known as the 'B' virus from the initials of this patient. Many similar cases have been reported since then. Herpesvirus simiae infects monkeys in the same manner that herpes simplex infects man, the infection being usually asymptomatic. The typical lesions produced are vesicles on the buccal mucosa, which ulcerate shedding the virus and infecting contacts. Though many human cases have followed monkey bites, in some, the infection was acquired by handling monkey tissues.

Herpesvirus simiae is similar to herpes simplex virus in its properties. The two are antigenically related but herpes simplex virus antibody does not protect against herpesvirus simiae infection. A formolised vaccine has been tried experimentally in laboratory workers at risk.

The disease in man is usually fatal. The rare

macular

patients who survive have serious neurological sequelae.

VARICELLA ZOSTER

It had been suggested by Von Bokay in 1909 that varicella (chickenpox) and herpes zoster are different manifestations of the same virus infection. Virological and epidemiological observations have proved this concept. The virus is, therefore, called varicella-zoster (V-Z) virus. Chickenpox follows primary infection in a non-immune individual, while herpes zoster is a reactivation of the latent virus when the immunity has fallen to ineffective levels. Thus, chickenpox is 'caught', but not zoster. Contact with zoster may lead to chickenpox, but contact with chickenpox will not lead to zoster.

The V-Z virus is similar to the herpes simplex virus in its morphology. It does not grow in experimental animals or chick embryos. The virus was first isolated in human embryonic tissue culture by Weller. It can be grown in cultures of human embryonic tissues, human amnion or HeLa cells. The cytopathic effects are similar to, but less marked than, those produced by herpes simplex virus. In cultures the virus remains cell associated and does not appear free in the medium. By using highly specific antiserum, it is possible to distinguish between herpes virus types 1, 2 and varicella-zoster viruses.

Varicella (chickenpox)

Chickenpox is one of the commonest and mildest of childhood infections. The disease may, however, occur at any age and adult chickenpox, which is more serious, is for some unknown reason rather common in some tropical areas.

The source of infection is a chickenpox or herpes zoster patient. Infectivity is maximum during the initial stages of the disease, when the virus is present abundantly in the upper respiratory tract. The buccal lesions which are characteristic of the early stage of the disease and the vesicular fluid are rich in virus content. Infectivity wanes as the disease progresses and the scabs are virtually noninfectious. There are no animal reservoirs of varicella.

The portal of entry of the virus is the respiratory tract. After an incubation period of about two weeks (7–23 days) the lesions begin to appear. In children, there is little prodromal illness and the disease is first noticed when skin lesions appear. In adults, prodromal fever and malaise with respiratory symptoms lasting for two or three days are common. The rash appears usually on the trunk. The evolution of the rash is so rapid that the various stages—macule, papule, vesicle, pustule and scab — cannot be readily followed in individual lesions. The rash of chickenpox differs from that of smallpox in a number of respects. In the former, the rash appears without prodromal illness, is centripetal in distribution, affecting mainly the trunk and sparing the distal parts of the limbs, and is very superficial without involving the deeper layers of the skin, resembling a drop of water lying on the skin. The chickenpox rash characteristically appears in crops during the first three or four days of the disease, so that lesions of varying age can be noticed on the same patient. It matures very quickly beginning to crust within 48 hours. These differences are, however, tendencies and may not apply in every case.

When varicella occurs in the adult, systemic symptoms may be severe, the rash very profuse and the entire disease much more intense than in children. The rash may become haemorrhagic and occasionally bullous lesions appear. Pitted scars on the skin may remain after recovery. Varicella pneumonia is more common in adults, and is usually fatal when it occurs in the elderly. In fatal cases of varicella pneumonia, an associated hepatitis may be found at autopsy. Varicella pneumonia may be followed by the appearance of scattered calcified nodules seen in the X-ray.

Secondary bacterial infections, usually due to staphylococci or streptococci, may occur. Reyes' syndrome—acute hepatic failure, encephalopathy and hypoglycaemia — may follow varicella, in some cases accompanied by a history of administration of large doses of salicylates. Varicella

encephalitis may occur which may be fatal or lead to brain damage.

Chickenpox is usually an uneventful disease and recovery is the rule. One attack confers lasting immunity. If primary infection occurs during pregnancy, the virus may cross the placenta and infect the fetus. If it occurs in early gestation, it may result in cicatricizing lesions of extremities as well as neuromuscular disorders. Administration of corticosteroids in chickenpox patients carries the risk of varicella pneumonia.

Laboratory diagnosis: Diagnosis is usually clinical. Formerly laboratory aid used to be frequently sought to distinguish between chickenpox and modified smallpox. The practice then was to test for laboratory evidence of variola and to make a presumptive diagnosis of chickenpox by exclusion. Diagnosis may be made by demonstrating multinucleated giant cells and type A intranuclear inclusion bodies in smears prepared by scraping the base of early vesicles, (Tzanck smears) and stained with toluidine blue, Giemsa or Papanicolou stain. Electron microscopy of the vesicle fluid may demonstrate the virus with typical herpes morphology. Virus isolation can be attempted from the buccal or cutaneous lesions in the early stages by inoculating human amnion, human embryonic tissue, HeLa or Vero cells. Serological tests are difficult, though CF, neutralisation and gel diffusion precipitation tests can be done. A fluorescent antibody technique has been described for detection of varicella antigen in the vesicle fluid. A rapid and simple method of detecting virus antigen is by counterimmunoelectrophoresis against zoster convalescent serum.

Prophylaxis and treatment: A live varicella vaccine has been developed by Japanese workers by attenuating a strain of varicella virus (Oka strain) by serial passage in tissue culture. Given by subcutaneous injection it induces good antibody response and is reported to provide protection lasting for several years. It has been recommended for use in high risk children as those with leukaemia and immunodeficiencies. The vaccine is not available in India.

VZ virus immune globulin prepared from patients convalescing from zoster can be used to provide passive protection in immunocompromised children exposed to infection. It is not useful in treatment.

Vidarabine and intravenous acyclovir have been reported to be of value in treatment.

Herpes zoster

(Shingles: Zona, *Herpein*, meaning to creep; *zoster*, meaning girdle)

While varicella is typically a disease of childhood, herpes zoster is one of old age, being common after the age of fifty years. The disease may, however, occur at any age and zoster has been reported very rarely even in the newborn.

Herpes zoster occurs usually in persons who had chickenpox several years earlier. The virus is believed to remain latent in the sensory ganglia, somewhat like the prophage in lysogenic bacteria. The virus may leak out at times but is usually held in check by the residual antibody. Years after the initial infection, when the antibody has dropped to ineffective levels, the virus may be reactivated, triggered by some precipitating stimulus like irradiation, travel along the sensory nerve and produce zoster lesions on the area of skin or mucosa supplied by it. This reactivation is associated with inflammation of the nerve which accounts for the neuritic pain that often precedes the skin lesions. The rash is usually unilateral and confined to the area supplied by a single sensory ganglion. The commonest sites are the areas innervated by spinal cord segments D3 to L2 and the trigeminal nerve, particularly, its ophthalmic branch. The rash heals in about two weeks. Pain and paraesthesia of the affected area may persist for weeks or months. Other complications are lower motor neuron paralysis which sometimes ensues, meningoencephalitis and generalised zoster where the lesions are scattered widely, perhaps due to haematogenous dissemination of the virus. Herpes zoster ophthalmicus is a com-

mon and troublesome presentation. The Ramsay Hunt syndrome is a rare form of zoster affecting the facial nerve, with eruption on the tympanic membrane and the external auditory canal, and often a facial palsy.

Diagnosis is easily made clinically. Laboratory diagnosis is as for chicken pox.

Herpes zoster is believed to be a mode of evolutionary adaptation by the V-Z virus which is an obligate human parasite. In small communities, the susceptibles are completely eliminated by varicella infection in childhood, and, therefore, the ability of the virus to remain latent and reappear as zoster years later confers on it a great survival advantage.

CYTOMEGALOVIRUSES

Large, inclusion bearing cells were reported as early as 1904 in the kidneys, lungs and liver of infants presumed to have died of congenital syphilis. They were mistaken for amoebae and were called *Entamoeba mortinatalium*. Goodpasture and Talbot, and Lipschutz thought them to be inclusion bodies and coined the term 'cytomegaly' for the condition. Farber and Wolbach recognised its appearance in the salivary glands of some children at autopsy. Hence the causative agent was called *salivary gland virus*. The name cytomegalovirus was suggested in 1960 by Weller, Hanshaw and Scott.

The virus exhibits strict host specificity and infection both *in vivo* and *in vitro* can be established only in the homologous species. Cytomegaloviruses have been identified in man, monkey, guinea pig and some other species. Human cytomegaloviruses can be grown in human fibroblast cultures. Epithelial cell cultures are not susceptible though epithelial cells are affected *in vivo*. Cultures have to be incubated for prolonged periods, upto 50 days as the cytopathic effects are slow in appearance.

Three serotypes have been recognised by the virus neutralisation test. They share complement fixing antigens. The virus is so ubiquitous and so persistently shed by cases and carriers, that to assign an aetiological role even in the presence of clinical symptoms is difficult. About ten per cent of healthy women may carry the virus in the uterine cervix.

Clinical features: Cytomegalovirus disease is rare but infection with the virus is extremely common. As with herpes simplex, the large majority of infections are inapparent, leading to prolonged latency. Clinical disease may be caused by either intrauterine or postnatal infections.

Intrauterine infection leads to the cytomegalic inclusion disease of the newborn which is often fatal. This is a generalised infection associated with hepatosplenomegaly, jaundice, thrombocytopaenic purpura and haemolytic anaemia. The cytomegalic inclusion disease is probably the most important cause of microcephaly. Other manifestations include chorioretinitis and cerebral calcification resembling congenital toxoplasmosis. Survivors may show mental retardation. Primary CMV infection in pregnancy is more likely to lead to congenital disease in the baby than recurrent infection in women who had been infected earlier.

Postnatal infections are usually inapparent, but may sometimes lead to insidious hepatitis or pneumonitis. Sometimes a syndrome resembling infectious mononucleosis may occur. This is particularly seen in persons receiving massive blood transfusions (posttransfusion mononucleosis). Patients with malignancies or immunodeficiencies and transplant recipients may develop pneumonitis, hepatitis or generalised disease with CMV, caused by reactivation of latent infection or by exogenous infection.

Laboratory diagnosis: Diagnosis may be established by recovery of the virus from the urine, saliva or other body fluids by inoculating human fibroblast cultures. A simpler, but less reliable, technique is the demonstration of cytomegalic cells in the centrifuged deposits from urine or saliva.

Epidemiology: CMV spreads slowly and proba-

bly requires close contact for transmission. It may spread through salivary secretions or venereally. A special method of transmission is by blood transfusion. The virus has been detected in saliva, urine, cervical secretions, semen, blood and milk. Congenitally infected infants have viruria upto 4–5 years. They are highly infectious in early infancy. Complement fixing antibodies are seen in 20–80 per cent of adult population indicating the high prevalence of infection.

EPSTEIN-BARR VIRUS

In 1958, Burkitt described an unusual lymphoma among children in certain parts of Africa and suggested on epidemiological grounds that the tumour may be caused by a mosquito borne virus. This led to several attempts at isolating viruses from such tumour tissues. A number of different viruses, apparently 'passenger viruses' were isolated from cultured lymphoma cells. One of these was a new type of herpesvirus named EB virus after the discoverers Epstein and Barr. The EB virus specifically affects cells of the B lymphocyte lineage. Only human and some subhuman primate B cells have receptors for the virus. EBV infected B cells are transformed so that they become capable of continuous growth *in vitro*.

EBV is ubiquitous in all human populations. By the age of three years, about 80 per cent of children in the affluent countries and about 99 per cent in the developing countries acquire EBV infection. Once infected, the virus is present in the individual for life. In most cases the infection is silent. The following clinical manifestations may result from EBV infection:

1. infectious mononucleosis;
2. lymphomas in graft recipients and immunodefectives;
3. Burkitt's lymphoma which occurs in two forms — the endemic or African type which is almost always associated with EBV infection, and the sporadic type in which only about 20 per cent show evidence of EBV infection.
4. Undifferentiated nasopharyngeal carcinoma seen mainly in males of Chinese origin in South East Asia and in East Africa.
5. It has been suggested but not proved that EBV infection can cause the 'chronic fatigue syndrome'.

Infectious mononucleosis *(Glandular fever)*

This is an acute, self-limited disease usually affecting young adults and characterised by fever, sore throat, lymphadenopathy and the presence of abnormal lymphocytes in peripheral blood. There is often an associated hepatitis, but it is usually subclinical and demonstrable only by liver function tests. Infection is believed to enter through the respiratory route by close contact with patients. The common mode of transmission is also believed to be by kissing and hence the disease is most prevalent among the adolescents and young adults ('kissing disease').

Laboratory diagnosis: The examination of blood during the initial phase may show a leucopenia

TABLE 52.1

Differential absorption test for Paul-Bunnel antibody

	Result of absorption with	
	Guinea pig kidney	Ox red cells
Normal serum	Absorbed	Not absorbed
Antibody after serum therapy	Absorbed	Absorbed
Infectious mononucleosis	Not Absorbed	Absorbed

due to a drop in the number of polymorphs. Later there is a prominent leucocytosis with the appearance of abnormal mononuclear cells characterised by deeply basophilic vacuolated cytoplasm and kidney shaped nuclei showing a lattice of fenestrated chromatin. These atypical mononuclear cells are not virus infected B lymphocytes, but lymphoblasts derived from T cells reactive to the virus infection. The blood picture may sometimes resemble lymphatic leukaemia.

The standard diagnostic procedure is the Paul Bunnel test. During infectious mononucleosis, heterophile antibodies appear in the serum of the patient. These antibodies agglutinate sheep erythrocytes. However, such antibodies may occur also after injections of sera and even sometimes in normal individuals. Infectious mononucleosis antibody may be differentiated by absorption tests. Inactivated serum (56°C for 30 minutes) in doubling dilutions is mixed with equal volumes of a 1% suspension of sheep erythrocytes. After incubation at 37°C for four hours the tubes are examined for agglutination. An agglutination titre of 100 or above is suggestive of infectious mononucleosis. For confirmation differential absorption of agglutinins with guinea pig kidney and ox red cells is necessary. Forssman antibody induced by injection of horse serum is removed by treatment with guinea pig kidney and ox red cells. Normally occurring agglutinins are removed by guinea pig kidney, but not by ox red cells. Infectious mononucleosis antibody is removed by ox red cells, but not guinea pig kidney. The Paul-Bunnel antibody develops early during the course of infectious mononucleosis, and disappears within about two months.

Tests have been described for the demonstration of specific antibody to EB virus by immunofluorescence, CF and gel diffusion. Specific antibody to EB virus lasts for very long periods after recovery and hence is not diagnostic of current infection. EB virus cannot be grown by conventional methods, but only on the cultures of Burkitt's lymphoma cells or lymphocytes from infectious mononucleosis cases.

There seems to be more than one aetiological variety of infectious mononucleosis. A number of cases that present the typical clinical picture of infectious mononucleosis do not develop Paul-Bunnel antibodies. Such cases are more commonly seen in India and may be due to cytomegalovirus or other unidentified agents.

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53 Adenoviruses

Adenoviruses are a group of medium sized, nonenveloped DNA viruses that share a common complement fixing antigen. They infect man, animals and birds, showing strict host specificity.

In 1953, Rowe and associates grew surgically removed human adenoid tissue in plasma clot cultures and noticed that the epithelial outgrowths underwent spontaneous degeneration resembling viral cytopathic change. This was neutralised by human sera. A viral agent was shown to be responsible for this degeneration. This was the prototype of the group of viruses subsequently designated 'adenoviruses' because they were originally isolated from the adenoids. Hilleman in 1954 isolated a related virus from throat washings of military recruits with acute respiratory illness. Adenoviruses have since been shown to be responsible for infections of the respiratory tract, eyes and perhaps of the intestine. The observation that some adenoviruses, inoculated into baby hamsters, produce sarcoma had led to studies on the possible role of adenoviruses in malignancy. However, there is no evidence at all relating adenoviruses to human cancer.

Morphology: Adenoviruses have a characteristic morphology. The capsid is composed of 252 capsomers arranged as an icosahedron with 20 triangular facets and 12 vertices. Of the 252 capsomers, 240 have six neighbours and are called *hexons* while the 12 capsomers at the vertices have five neighbours and are called *pentons*. Each penton unit consists of a penton base anchored in the capsid and a projection consisting of a rod-like portion with a knob attached at the distal end. The latter is known as *fiber*. Thus, the virion has the appearance of a space vehicle.

Resistance: Adenoviruses are relatively stable, remaining viable for about a week at 37°C. They are readily inactivated at 50°C. They resist ether and bile salts.

Growth and host range: Adenoviruses are host specific and so laboratory animals are not susceptible to adenoviruses infecting man. Human adenoviruses grow only in tissue cultures of human origin, such as human amnion, HeLa or HEP-2. Cytopathic changes may take several days to develop and consist of cell rounding and aggregation into grape-like clusters. Infected cells swell and become ballooned. Intranuclear inclusions may be seen in stained preparations.

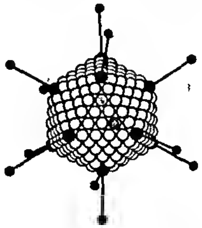

Fig. 53.1 Morphology of adenovirus

Classification: The family *Adenoviridae* contains two genera, *Mastadenovirus*, the adenovirus of mammals and *Aviadenovirus*, the adenovirus of birds. Mastadenoviruses have been isolated from man (41), monkey (24), cow (8), horse (1), sheep (3), dog (2), pig (4), mouse (2) and opossum (1). Figures in brackets show the number of serotypes isolated. Aviadenoviruses have been isolated from fowls (9), goose (3) and turkey (2). They infect only the homologous species, with the exception of oncogenic human adenoviruses (e.g., types, 12, 18, 31) that cause sarcomas when injected into newborn hamsters.

All adenoviruses share a common complement fixing antigen. Serotypes are distinguished by neutralisation test. Human adenoviruses are classified into four subgroups based on haemagglutination and other properties (Table 53.1).

Pathogenesis: Adenoviruses cause infections of the respiratory tract, eye, bladder and intestine. More than one type of virus may produce the same clinical syndrome and one type of virus may cause clinically different diseases. The following syndromes have been recognised.

Pharyngitis: Adenoviruses are the major cause of nonbacterial pharyngitis and tonsillitis. Several types may cause the condition. Types 1 – 7 are commonly isolated.

Pneumonia: Adenovirus types 4 and 7 are associated with pneumonia in adults resembling primary atypical pneumonia. In infants and young children type 7a may lead to more serious and even fatal pneumonia.

Acute respiratory diseases (ARD): This occurs usually as outbreaks in military recruits. Serotypes 4, 7 and 21 are the agents commonly isolated.

Pharyngoconjunctival fever: This syndrome of febrile pharyngitis and conjunctivitis seen in civilian population is usually associated with serotypes 3 and 7.

Epidemic keratoconjunctivitis (EKC): This is a serious epidemic disease seen mainly in industrial workers exposed to dust (shipyard eye). The usual aetiological agent is type 8. Types 19 and 37 have also been responsible for EKC in recent years.

Acute follicular conjunctivitis: This is a nonpurulent inflammation of the conjunctiva with enlargement of the submucous lymphoid follicles and of the preauricular lymph nodes. Types 3 and 7a are commonly responsible.

Acute haemorrhagic cystitis in children and *generalised exanthem* are two other syndromes which have been reported. Adenoviruses types 11 and 21 are responsible for the former.

Diarrhoea: Adenoviruses can often be isolated from faeces, but their relation to any intestinal disease has not been conclusively established.

TABLE 53.1

Classification of human adenoviruses

Subgroup	Serotypes	Haemagglutination	Oncogenicity
A	12, 18, 31	Nil	High
B	3, 7, 11, 14, 16, 21	Monkey + Rat ∓	Weak
C	1, 2, 4, 5, 6	Monkey – Rat ±	Nil, but can transform cells.
D	8, 9, 10, 13, 15, 17, 19, 20, 22-28	Monkey – Rat +	Nil, but can transform cells.

However, some fastidious adenoviruses, which can be demonstrated abundantly in faeces by electron microscopy but fail to grow in conventional tissue cultures, can cause diarrhoeal disease in children (e.g., types 40, 41). They have been designated 'enteric type' adenoviruses. Special techniques of tissue culture (use of trypsinised monkey kidney cells or transformed human embryonic kidney cells) have been developed for their cultivation.

Adenoviruses have been isolated from mesenteric lymph nodes in cases of *mesenteric adenitis* and *intussusception* in children.

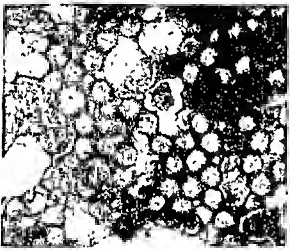

Fig. 53.2 Adenoviruses. Stools from a child with diarrhoea. Virus particles show characteristic hexagonal shape × 200,000. Negative stain with 3 per cent ammonium molybdate of faecal sediment (Courtesy Prof. M. Mathen, Christian Medical College, Vellore)

Laboratory diagnosis: Diagnosis can be established by isolation of the virus from the throat, eye, urine or faeces. The materials are inoculated in tissue cultures. Preliminary identification is possible by noting the cytopathic effects and by complement fixation tests with adenovirus antiserum. By haemagglutination with rat and monkey erythrocytes, the isolate can be classified into subgroups. Typing is done by neutralisation tests.

For serological diagnosis, rise in titre of antibodies should be demonstrated in paired sera. Examination of a single sample of serum is inconclusive as adenovirus antibodies are so common in the population.

Prophylaxis. Because of the generally mild nature of adenovirus infections and the existence of several serotypes, vaccines are neither indicated nor practicable. Experimental vaccines incorporating types 3, 4 and 7 produced in monkey kidney cells proved useful against acute respiratory disease in military recruits. But the oncogenic potential of adenoviruses and of the SV40 virus often occurring as a contaminant of monkey kidney cells has caused these to be withdrawn. Vaccination with attenuated strains and oral administration of a live virus enclosed in capsules have been tried with success. The present trend is to use adenovirus structural proteins for induction of neutralising antibodies.

ADENOVIRUS-ASSOCIATED VIRUSES (AAV)

Electron microscopy of several adenovirus preparations have revealed small icosahedral viral particles, 20-25 nm in diameter. These viral particles are unable to multiply except in cells simultaneously infected with adenoviruses and are called adeno-associated viruses (AAV) or adenosatellite viruses. They can be detected by electron microscopy and complement fixation or immunofluorescence with specific antisera. Types 1, 2 and 3 are of human origin and cause natural infection while type 4 is of simian origin. Their pathogenic role is uncertain. They have been classifed in the genus *Dependovirus* in the *Parvovirus* family.

Further Reading

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54 Picornaviruses

The picornavirus group comprises a large number of very small (*pico*, meaning small) RNA viruses. They are nonenveloped viruses, 20–30 nm in size, resistant to ether and other lipid solvents. Picornaviruses of medical importance are the enteroviruses that parasitise the enteric tract and the rhinoviruses that infect the nasal mucosa. Foot and mouth disease virus and many other viruses that infect animals also belong to the picornavirus group.

ENTEROVIRUSES

Enteroviruses of medical importance include: Polioviruses types 1–3, Coxsackievirus A-types 1–24 (type 23 same as ECHO7), Coxsackievirus B-types 1-6, Echoviruses types 1–34 (ECHO10 is now classified as Reovirus 1 and ECHO28 as Rhinovirus 1A) and Enteroviruses types 68–72.

Poliomyelitis has been recognised from very early times as a paralytic disease of children (infantile paralysis). But it was only towards the end of the 19th century that it was characterised as a separate clinical entity capable of causing infections in which paralytic cases are far outnumbered by silent inapparent infections. Landsteiner and Popper (1909) reported experimental transmission of the disease to monkeys by intracerebral inoculation of the spinal cord from fatal cases of poliomyelitis. The experimental study of the disease was restricted as monkeys were the only laboratory animals susceptible to the virus. Armstrong (1939) succeeded in adapting a poliovirus (type 2 Lansing strain) to cotton rats and mice but not all strains could be thus adapted. Progress was also inhibited by the dogma then held that polioviruses were strictly neurotropic, multiplying only in neural tissues. The demonstration by Enders, Weller and Robbins (1949) that polioviruses could grow in cultures of non-neural cells from human embryos, producing cytopathic effects, was a major breakthrough. The Nobel Prize was awarded to them in recognition of the seminal importance of this discovery in the development of virology as a whole.

A new type of virus was isolated by Dalldorf and Sickles (1948) from the faeces of children with paralytic poliomyelitis, from whom type 1 poliovirus was also isolated. The virus caused paralysis on inoculation into suckling mice. This was called 'coxsackievirus', as the patients came from the village of Coxsackie in New York. Many similar viruses have since been isolated from faeces and throats of patients with different diseases as well as from healthy individuals. They have been designated coxsackieviruses, classified into groups A and B based on the pathological changes produced in suckling mice.

The introduction of tissue culture techniques in diagnostic virology led to the isolation of several cytopathogenic viruses from faeces of sick as well as healthy persons. They were called 'orphan' viruses as they could not be associated with any particular clinical disease. They are now known by the descriptive term '*e*nteric *c*ytopathogenic *h*uman *o*rphan (ECHO) viruses'. Several 'orphan' viruses have been isolated from animal faeces also.

The classification of enteroviruses as coxsackieviruses and echoviruses is not satisfactory, but

the names have become so well entrenched that they cannot be changed. It was, therefore, decided that from 1969, any new enterovirus identified would not be classified into either of these groups but would be merely assigned enterovirus type numbers, starting with type 68 (because 67 types of enteroviruses had already been classified by then, consisting of three polio, 30 coxsackie and 34 echoviruses). Hence we have now five new enterovirus types 68–72. Enterovirus 72 is the virus causing infectious hepatitis (Hepatitis type A). Because of its special status, it is considered in the chapter on Hepatitis viruses.

Most enteroviruses are host specific, infecting only one or a few related species. There is no common group antigen for enteroviruses, though some of them show antigenic cross reactions.

Polioviruses

Morphology: The virion is a spherical particle, about 30 nm in diameter composed of capsomers arranged in icosahedral symmetry. The virus can be crystallised, and arrays of virus crystals can be seen in the cytoplasm of infected cells.

Resistance: Poliovirus is resistant to ether, chloroform, bile, proteolytic enzymes of the intestinal contents and detergents. It is stable at pH 3. It can survive for months at 4°C and years at -20°C. Depending on conditions like temperature, moisture, pH and amount of virus, its survival in faeces at room temperature may vary from a day to several weeks. It is readily inactivated by heat (55°C for 30 minutes). Molar $MgCl_2$ protects the virus against heat inactivation. Formaldehyde and oxidising disinfectants destroy the virus. Chlorination destroys the virus in water but organic matter delays inactivation. Phenolic disinfectants are not effective. Poliovirus does not survive lyophilisation well.

Antigenic properties: By neutralisation test, the poliovirus strains have been classified into three types, 1, 2 and 3. Type 1 is the commonest and causes most epidemics. Intratypic strain difference can be demonstrated by refinements in neutralisation tests, but this is not significant in immunity against the disease.

By complement fixation or precipitation tests, two antigens C and D (D = dense; C = coreless or capsid) can be recognised. The D antigen, also called the Native or N antigen, is associated with the whole virion and is type specific. The C antigen, also called the Heated or H antigen associated with the 'empty' noninfectious virus, is less specific and reacts with heterotypic sera. The D antigen is converted into the C antigen by heating the virus at 56° C. Anti-D antibody is protective and therefore the potency of injectable poliovaccine is measured in terms of D antigen units. Anti-C antibody does not neutralise virus infectivity.

Host range and cultivation. Natural infection occurs only in man. Experimentally, monkeys may be infected by intracerebral or intraspinal inoculation. Chimpanzees and cynomolgus monkeys may be infected orally also. Some established strains have been adapted for growth in rodents and chick embryos, but fresh isolates will not infect them.

The virus grows readily in a variety of tissue cultures of primate origin. Primary monkey kidney cultures are used for diagnostic cultures and vaccine production. The infected cells round up and become refractile and pyknotic. Eosinophilic intranuclear inclusion bodies may be demonstrated in stained preparations. Well formed plaques develop in infected monolayers with agar overlay.

Pathogenesis: The virus enters the body by ingestion. Inhalation of droplets can also be a mode of entry in close contacts of patients in the early stage of the disease. The virus multiplies in the lymphatic tissues of the alimentary canal, from the tonsils to the Peyer's patches, and enters the regional lymphatics. After multiplication in these sites, the virus enters the bloodstream and is carried to the spinal cord and brain. Direct neural

transmission to the central nervous system may also occur under special circumstances as in poliomyelitis following tonsillectomy.

Following inapparent infection and vaccination with avirulent strains, infection is limited to the intestinal lymphatics usually and viraemia may not occur. This, however, is sufficient for the development of immunity.

In the central nervous system, the virus multiplies selectively in the neurons and destroys them. The earliest change is the degeneration of Nissl bodies (chromatolysis). Nuclear changes follow. When degeneration becomes irreversible, the necrotic cell lyses or is phagocytosed by leucocytes or macrophages. Lesions are mostly in the anterior horns of the spinal cord but the posterior horns and intermediate columns may also be involved to some extent. Pathological changes are usually more extensive than the distribution of paralysis. In some cases, extensive encephalitis occurs involving primarily the brainstem, but extending upto the motor and premotor areas of the cerebral cortex.

Clinical features: The incubation period is on an average about 10 days, but may range from four days to four weeks. The earliest manifestations are associated with the phase of viraemia and consist of fever, headache, sore throat and malaise lasting 1–5 days. This is called the 'minor illness' and in many cases may be the only illness (abortive poliomyelitis). If the infection progresses, the minor illness *is followed 3–4 days later by* the 'major illness'. The fever comes on again (biphasic fever), along with headache, stiff neck and other evidences of meningitis. This marks the stage of viral invasion of the central nervous system. Sometimes the disease does not progress beyond this stage of aseptic meningitis (nonparalytic poliomyelitis). In those proceeding to the paralytic stage, flaccid paralysis develops. Paralysis is focal in distribution initially, but spreads over the next 3–4 days. Depending on the distribution of paralysis, cases are classified as spinal, bulbar or bulbospinal. Mortality ranges from 5–10 per cent and is mainly due to respiratory failure. Recovery of the paralysed muscles takes place in the next 4–8 weeks and is usually complete after six months, leaving behind varying degrees of residual paralysis.

Laboratory diagnosis: Unlike other enteroviruses, poliovirus can seldom be isolated from the CSF, but can be obtained from the spinal cord and brain, postmortem. The virus can be isolated from the throat in the early stage of the disease and from the faeces throughout the course of the disease and during convalescence. After appropriate processing to destroy bacteria (centrifugation, treatment with ether, addition of antibiotics), specimens are inoculated into tissue culture. Primary monkey kidney cells are usually employed, though any other human or simian cell culture may be used. The virus growth is indicated by typical cytopathic effects in 2–3 days. Identification is made by neutralisation tests with pooled and specific antisera. It must be remembered that mere isolation of poliovirus from faeces does not constitute a diagnosis of paralytic poliomyelitis as symptomless infections are so common. Virus isolation must be interpreted along with clinical evidence.

Serodiagnosis is less often employed. The antibody rise can be demonstrated in paired sera by neutralisation or complement fixation tests. Antibodies appear soon after the onset of paralysis so that even the first sample of serum may contain appreciable amounts of antibody. *Neutralising antibodies appear early and persist* for life. In CF test antibodies to C antigen appear first and disappear in a few months, while anti-D antibodies take some weeks to appear after infection but last for five years. The CF test is useful to identify exposure to poliovirus but not for type specific diagnosis.

Prophylaxis: Passive immunisation by administration of human gamma globulin is of limited value and is restricted to special cases at high risk as in pregnant women exposed to infection.

Attempts at active immunisation with vaccines date from 1910, soon after the discovery of

poliovirus. The early vaccines were crude suspensions of the spinal cord from infected monkeys, inactivated with formalin (Brodie and Park) or ricinoleate (Kolmer). They were not only ineffective but often dangerous, leading to vaccination poliomyelitis. Polio vaccines, therefore, became unpopular. It was only after 1949, when tissue culture was used for growing the virus and the existence of three antigenic types of polioviruses was recognised, that fresh developments in vaccine preparation became possible. By 1953, Salk developed a killed vaccine. Almost simultaneously, Koprowsky, Cox and Sabin independently developed live attenuated vaccines.

Salk's killed polio vaccine is a formalin inactivated preparation of the three types of the poliovirus grown in monkey kidney tissue culture. Standard virulent strains are used. The individual types of polioviruses are grown separately in monkey kidney cells. Viral pools of adequate titre are filtered to remove cell debris and clumps, and inactivated with formalin (1:4000) at 37° C for 12 – 15 days. Stringent tests are carried out to ensure complete inactivation and freedom from extraneous agents. The three types are then pooled and, after further tests for safety and potency, are issued for use.

A nationwide controlled field trial conducted in 1954 in the USA under the direction of Francis confirmed the safety of the Salk vaccine and showed that it gave 80–90 per cent protection against paralytic poliomyelitis. However, in 1955, an unfortunate incident happened that led to doubts about the safety of the vaccine. Over 100 cases of paralytic poliomyelitis occurred in the vaccinees and their contacts following use of an insufficiently inactivated vaccine. This 'Cutter incident' was investigated in detail and led to the introduction of further safeguards. The vaccine, after these modifications, has been completely safe

Killed vaccine is given by injection. Three doses given 4–6 weeks apart constitute the primary vaccination, to be followed by a booster to be given six months later. The first dose should be given to babies after the age of six months to ensure that the antibody response is not impaired by residual maternal antibodies. Immunity can be sustained by booster doses every 3–5 years thereafter.

Live polio vaccines were developed independently by Koprowsky, Cox and Sabin. Koprowsky, in 1952, reported vaccination of children with a strain of poliovirus type 2 attenuated by repeated passage in cotton rats. Subsequently, attenuated strains were selected by growth in tissue culture. All three vaccines were used initially, but now only Sabin's attenuated strains are employed These have been obtained by plaque selection in monkey kidney tissue culture. Attenuated strains for live vaccine should possess the following criteria: 1) They should not be neurovirulent as tested by intraspinal inoculation in monkeys; 2) they should be able to set up intestinal infection following feeding and should induce an immune response; 3) they should be stable and should not acquire neurovirulence after serial enteric passage; 4) they should possess stable genetic characteristics (markers) by which they can be differentiated from the wild virulent strains.

Several markers have been described for differentiating the wild from the attenuated strains. The following markers are commonly used: 1) *d* marker: wild strains will grow well in low levels of bicarbonate, but avirulent strains will not; 2) *rct 40:* wild strains grow well at 40° C while avirulent strains grow poorly; 3) *MS:* wild strains grow well in stable cell line of monkey kidney while avirulent strains grow poorly; and 4) McBride's intratypic antigenic marker shown by the rate of inactivation by specific antiserum.

Live polio vaccine is prepared by growing the attenuated strains in monkey kidney cells. Very stringent precautions are taken to ensure freedom from extraneous agents like SV 40 and B virus. After tests for neurovirulence, genetic stability and potency, the vaccine is issued either in the monovalent or trivalent form, in pleasantly flavoured syrup or candy. The use of molar $MgCl_2$ or sucrose stabilises the vaccine against heat inactivation, particularly under tropical con-

ditions. The vaccine is usually given in the trivalent form. It can be given to infants, as the maternal antibody has little effect on intestinal infection. Theoretically, a single dose should be sufficient to establish infection and immunity but in practice three doses are given at 6–8 week intervals, to ensure that all three types of the vaccine virus multiply in the intestine, overcoming interference among themselves and with other enteric viruses. It has been recommended that, in the tropics, the number of doses of vaccine be increased to five, in order to ensure seroconversion in the vaccinees.

Live oral poliovaccine used in India is stated to contain Type 1 virus 10 lakh, Type 2 virus 2 lakh and Type 3 virus 3 lakh TC ID50 per dose (0.5 ml). The liquid vaccine is thermostabilised with $MgCl_2$ which acts only at a pH below 7.0. To maintain the pH, the vaccine has to be kept in air-tight containers. The shelf life of the vaccine at 4–8°C is four months and at –20°C is two years.

There has been much controversy about the relative merits of killed and live vaccines. They may be considered under the following headings:

1 *Safety:* Both vaccines are safe. It has been suggested that the attenuated strains tend to acquire neurovirulence on serial enteric passage as may happen following vaccination. A few cases of vaccine induced poliomyelitis have been reported but the incidence is so low (one in several million) that the risk is negligible. Over a billion doses of live vaccine have been administered by now in different parts of the world. The live vaccine is not safe in the immunodeficient or immunosuppressed subjects, but the killed vaccine will do no harm.

2 *Efficiency:* A full course of killed vaccine induces a satisfactory immune response. One or two doses of live vaccines have produced 90–100 per cent seroconversion in children in the advanced countries. But in the developing countries in the tropics, the response has not been so satisfactory. This is especially so with polio type 1, more than half the vaccinees failing to show serological response after two or three doses. The reason for this disparity is not certain, though several possibilities have been suggested. Intestinal carriage of enteroviruses is common in the tropics and this may prevent infection by the vaccine virus by interference. Experimentally, it has been shown that coxsackie B viruses may interfere with poliovirus while coxsackie A may be synergistic. Diarrhoeal diseases are highly prevalent. Increased intestinal motility prevents colonisation by the vaccine virus. Failure of oral vaccination may also be due to the practice of breast feeding immediately before or after the vaccine is given. The vaccine virus may be neutralised by antibodies in the breast milk or by some other inhibitory agent in the intestinal secretions. An inhibitor of poliovirus has been identified in saliva. This can be neutralised by horse antiserum to human gamma globulin. Because of the poor rate of seroconversion after oral immunisation in India and other tropical areas, primary immunisation with killed vaccines (given along with the triple antigen) may become necessary. This may be followed by live oral vaccines for achieving intestinal immunity.

3 *Ease of administration* Live vaccine given orally is obviously preferable to killed vaccine given by injection. An advantage of the killed vaccine is that it can be administered along with the DPT vaccine as a 'quadruple vaccine'.

4 *Economy:* Live vaccine is very much more economical. This is an important aspect in mass vaccination campaigns in the developing countries.

5 *Nature of immunity:* This is perhaps the most important point of difference between the two. Killed vaccine induces only systemic antibody response. There is no intestinal immunity, so that even in the vaccinated, infection with a wild strain may lead to intestinal multiplication and dissemination of the virus. The individual is protected by the circulating antibodies. Live vaccine, on the other hand, induces local immunity in the

gut so that wild viruses are unable to multiply in the intestines. Hence, it protects the individual and the community.

6 *Duration of immunity:* Immunity following killed vaccine may need to be maintained by booster doses periodically, while immunity following live vaccine resembles natural active immunity in being lifelong.

7 *Use in epidemics:* Communitywide administration of live vaccine, ideally monovalent vaccine of the same type as that causing the epidemic, early during an epidemic of paralytic poliomyelitis can stop the epidemic. This has been successfully practised in different parts of the world.

8 *Spread of vaccine virus in the community:* The tendency of the vaccine virus to spread naturally in the community, especially among children, was considered a disadvantage. Actually, it may even be beneficial and may help to extend the vaccine coverage in a community. Ideally, however, it is desirable to vaccinate the whole community at one time so that natural dissemination is prevented. The strategy of administering the vaccine to all children in a region on the same day (pulse immunisation) has been found to be useful in the developing countries.

9 *Eradication of poliomyelitis:* By global immunisation with live vaccine, it is possible to eradicate the disease. The World Health Organisation Assembly at its 15th Planning meeting on 13th May, 1988, has passed a resolution committing the WHO to global eradication of poliomyelitis by the year 2000.

Epidemiology: Poliomyelitis is an exclusively human disease and the only source of virus is man, the patient or much more commonly the symptomless carrier. Patients shed the virus in faeces for varying periods, about 50 per cent for three weeks and a small proportion for 3–4 months. No permanent carriers occur. While faecal virus is the important source of dissemination in the community, virus shed in throat secretions during the early part of the disease seems to be an equally important source of infection for the contacts of patients.

Infection is, in general, asymptomatic. The ratio of subclinical to clinical infections has been stated as 100 or 1000 to 1. The outcome of infection is influenced by the virulence of the infecting strain, the dose of infection and the age of the individual, adults being more susceptible than children. The following factors may predispose to the incidence of paralysis: 1) Pregnancy carries an increased risk of paralysis, perhaps due to the associated hormonal changes. 2) Tonsillectomy during the incubation period may lead to bulbar poliomyelitis. 3) Injections such as triple vaccine, especially alum containing preparations, may lead to paralysis involving the inoculated limb. The mechanism is uncertain. The trauma may lead to virus entry into local nerve fibres, or the segment of spinal cord corresponding to the site may be more susceptible to viral damage due to reactive heperaemia. 4) Severe muscular exertion or trauma during the preparalytic stage increases the risk of paralysis.

Poliovirus type 1 is responsible for most epidemics of paralytic poliomyelitis. Type 3 also causes epidemics to a lesser extent. Type 2 usually causes inapparent infections in the western countries, but in India paralysis due to type 2 is quite common. Immunity is type specific, but there is significant amount of cruss protection between types 1 and 2, between types 2 and 3, and little or none between types 1 and 3.

Poliomyelitis is worldwide in distribution. Originally, the epidemiology of the disease was similar in all countries and paralysis was seen exclusively in children (infantile paralysis). With improvement of living conditions and sanitation in the advanced countries, the age distribution changed and paralytic cases in adults became more common. This was due to a lack of natural contact with the virus during childhood so that a population of adults grew up without immunity to the disease. By systematic immunisation with either live or killed vaccine, many affluent coun-

tries have achieved virtual eradication of the disease. In the developing nations, the situation has not changed. In India and other tropical countries, about 90 per cent of children acquire antibodies to all three types of poliovirus by the age of five years. Over 80 per cent of all paralytic cases occur before the age of three years and only very occasionally does a case occur after the first decade of life. The incidence of paralytic poliomyelitis in India is 20–40/100,000 population per year. Every two minutes a child becomes paralysed making a total of 720 every day. An estimated 200,000 children develop paralytic poliomyelitis annually in India, more than in all the rest of the world put together. The disease is equally prevalent in rural and urban areas.

The epidemiology of poliomyelitis has undergone marked changes during the last three decades with the use of polio vaccines. Immunisation provides a tool for eradicating the disease altogether. But the elimination of the disease from a community or a nation brings on the responsibility of maintaining the immunity by a continuing programme of surveillance and vaccination of new entrants, lest the absence of natural immunisation in childhood should produce a generation highly susceptible to the disease

Coxsackieviruses

The prototype strain was isolated by Dalldorf and Sickles (1948) from Coxsackie village in New York. Several related viruses have been isolated since then from different parts of the world. The characteristic feature of this group is their ability to infect suckling, but not adult mice. Based on the pathological changes produced in suckling mice, coxsackieviruses are classified into two groups, A and B.

Properties of the virus: Coxsackieviruses are typical picornaviruses. Following inoculation in suckling mice, group A viruses produce a generalised myositis and flaccid paralysis leading to death within a week. Group B viruses produce a patchy focal myositis, spastic paralysis, necrosis of the brown fat and, often, pancreatitis, hepatitis, myocarditis and encephalitis. By neutralisation tests, group A viruses are classified into 24 types and group B into six types. All types in the group B share a common complement fixing antigen. Coxsackie A 23 is the same as echo 9. Some coxsackieviruses (A 7,20,21,24 and B 1,3,5,6) agglutinate human or monkey erythrocytes.

Host range and growth: It is necessary to employ suckling mice for the isolation of coxsackieviruses. Inoculation is usually made by intracerebral, subcutaneous and intraperitoneal routes. Adult mice are not susceptible. Suckling hamsters can be infected experimentally.

All coxsackie B viruses grow well in monkey kidney tissue cultures, while in group A, only type 9 grows well. Some group A viruses grow in HeLa cells.

Clinical features: Coxsackieviruses produce a variety of clinical syndromes in man, ranging from trivial to fatal infections. The following types have been recognised.

1. Herpangina (vesicular pharyngitis) is a common clinical manifestation of coxsackie group A infection in children. It is a severe febrile pharyngitis, with headache, vomiting and pain in the abdomen. The characteristic lesions are small vesicles, on the fauces and posterior pharyngeal wall, that break down to form ulcers.

2. Aseptic meningitis may be caused by most group A and all group B viruses. A maculopapular rash may be present. The disease may sometimes occur as epidemics. Occasionally, the picture may resemble paralytic poliomyelitis. Russian workers had suggested that coxsackie A7 be classified as poliovirus type 4 because of its tendency to produce paralysis.

3. Epidemics of fever with maculopapular or vesicular rashes may be produced by types A9, 16 and 23. A vesicular lesion involving mouth, hands and feet can be caused by A 5, 10 or 16 (hand, foot and mouth disease).

4. Minor respiratory infections resembling common cold may be caused by A 10,21,24 and B3

5. Epidemic pleurodynia or Bornholm disease (so called because it was first described on the Danish island of Bornholm) is a febrile disease with stitch-like pain in the chest and abdomen, caused by group B viruses. The disease may occur as epidemics or sporadically.

6. Myocarditis and pericarditis in the newborn, associated with high fatality may be caused by group B viruses. The disease may sometimes occur in older children and adults also.

7. Juvenile diabetes has been reported to be associated with coxsackie B4 infection, but a causal role for this virus has not been established.

8. Orchitis due to coxsackievirus has also been reported

9. Transplacental and neonatal transmission has been demonstrated with coxsackie B viruses resulting in a serious disseminated disease that may include hepatitis, myocarditis, meningoencephalitis and adrenocortical involvement

Laboratory diagnosis: Virus isolation from the lesions or from faeces may be made by inoculation into suckling mice. Identification is by studying the histopathology in infected mice and by neutralisation tests. Due to the existence of several antigenic types, serodiagnosis is not practicable.

Epidemiology: Like other enteroviruses, coxsackieviruses inhabit the alimentary canal primarily and are spread by the faecal-oral route. Coxsackie B virus epidemics tend to occur every 2-5 years. Young infants are most commonly affected. Vaccination is not practicable as there are several serotypes and immunity is type specific.

Echoviruses

When tissue cultures became routine procedures in diagnostic virology, several cytopathogenic viruses came to be isolated from the faeces of sick as well as healthy individuals. These viruses were not pathogenic for laboratory animals. They were neutralised by pooled human gamma globulin. As they could not be associated with any particular clinical disease then, they were called 'orphans'. They have been given the descriptive designation 'enteric cytopathogenic human orphan viruses' and are generally known by the sigla 'echoviruses'.

Properties of the virus: Echoviruses resemble other picornaviruses in their properties. By neutralisation tests, they have been classified into 34 serotypes. Types 10 and 28 have been removed from the group, the former becoming reovirus and the latter a rhinovirus.

Some echoviruses (types 3,6,7,11,12,13,19, 20,21,24,29,30 and 33) agglutinate human erythrocytes. Haemagglutination is followed by elution, rendering the cells inagglutinable by echo or coxsackieviruses, but not by myxoviruses.

Growth and host range: All echoviruses grow well in human and simian kidney cultures producing cytopathic effects. Echoviruses infect only man naturally. They are not pathogenic to laboratory animals though occasional strains may produce paresis on inoculation into monkeys and newborn mice.

Resistance. The presence of 0.5 ppm of free chlorine inactivates in ten minutes the quantity of echoviruses commonly encountered in swimming pools. It is not inactivated by 70% ethanol and 5% lysol. It is resistant to 20% ether at 4°C for 18 hours and to deoxycholate.

Clinical features: Though echoviruses were originally considered to be 'orphans' they have since been shown to produce a variety of disease patterns. Most infections are asymptomatic. In general, the clinical features resemble those produced by coxsackieviruses. Fever with rash and aseptic meningitis, sometimes as epidemics, can be produced by several serotypes, predominantly by types 4, 6, 9 and 16. Echoviruses perhaps constitute the commonest cause of aseptic meningitis. Echoviruses have frequently been isolated from minor respiratory disease and gastroen-

teritis, but their aetiological role has not been proved. Paralysis, gastroenteritis and hepatic necrosis usually ending fatally have also been attributed to echoviruses.

Laboratory diagnosis: Faeces, throat swabs or CSF may be inoculated into monkey kidney tissue cultures and virus growth detected by cytopathic changes. The large number of serotypes makes identification by neutralisation tests laborious. This may be simplified by haemagglutination and the use of serum pools for neutralisation. Serological diagnosis is not practicable except in case of epidemics where the causative serotype has been identified.

Epidemiology: Like other enteroviruses, echoviruses inhabit the alimentary tract primarily and are spread by the faecal-oral route. Epidemics may occur, especially in summer. Vaccination has not been attempted.

New enterovirus types: Of the five new enterovirus types 68–72, type 68 was isolated from pharyngeal secretions of children with pneumonia and bronchitis, and type 71 from cases of meningitis and encephalitis. Type 69 is not associated with any human disease. Type 70 causes acute haemorhagic conjunctivitis. Type 72 is Hepatitis virus type A.

Acute haemorrhagic conjunctivitis

A pandemic of acute haemorrhagic conjunctivitis, apparently arising in West Africa in 1969 has spread widely involving several parts of Africa, India, South East Asia, Japan, England and Europe. The incubation period is about 24 hours and the symptoms are sudden swelling, congestion, watering and pain in the eyes. Subconjunctival haemorrhage is a characteristic feature. There is transient corneal involvement, but recovery is usually complete in 3–7 days.

TABLE 54.1

Clinical syndromes commonly caused by Enteroviruses

Syndrome	Poliovirus	Coxsackie A	Coxsackie B	Echo	New enterovirus types
Paralysis	1,2,3,	7	–	–	–
Aseptic meningitis	1,2,3	7,9,23	1–6	Several (2,3,4,6,7,9 11,14,16,17, 18,19,25,33)	71
Encephalitis	1,2,3	9	3–6	6,9,17,19	71
Fever with rash	–	9,16,23	–	4,6,9,16	–
Hand, foot and mouth disease	–	5,10,16	–	–	–
Herpangina	–	1–6,8,10	–	–	–
Upper respiratory infection	–	21	–	11,20	–
Pneumonitis, Bronchiolitis	–	–	–	–	68
Bornholm disease	–	–	1-5	–	–
Myocarditis, pericarditis	–	–	1-5	–	–
Acute haemorrhagic conjunctivitis	–	24	–	–	70
Hepatitis Type A					72

Radiculomyelopathy has been reported as a complication from India. Sometimes it leads to paralysis resembling poliomyelitis.

The causative agent has been identified as ~~enterovirus type 70~~. It grows only on cultured human cells (human embryonic kidney or HeLa) on primary isolation, but can be adapted to grow on monkey kidney cells. Coxsackievirus type A 24 also produces the same disease. Both these viruses show intratypic antigenic differences.

RHINOVIRUSES

Common cold is probably the commonest infectious disease of man. Bacteria free filtrates of nasal secretions from patients had been shown to transmit colds to human volunteers as early as 1914. Dochez (1938) reported similar transmission of colds to chimpanzees. From 1946, a group of workers under C.H. Andrewes had been investigating the disease using human volunteers at the Common Cold Research Unit, Salisbury, U.K. The common cold virus was isolated there by Tyrrell and his colleagues (1960) by inoculating specimens into monkey kidney tissue culture incubated on roller drums at 33°C instead of 37°C and using a medium containing a lower bicarbonate concentration than usual. A related virus had been described earlier independently by Price and by Pelon and colleagues (1956) and named JH and 2060, respectively. This was classified as echovirus type 28. These and several similar viruses from common cold cases reported thereafter by other workers were known as common cold viruses, Salisbury viruses or muriviruses, till the name *rhinovirus* was finally applied to this group.

Properties of the viruses: Rhinoviruses resemble other picornaviruses in size and structure. They differ from enteroviruses in being more acid labile, but more heat stable. By neutralisation tests, they have been classified into over 110 serotypes. Immunity is type specific.

Host range and growth: Apart from man, rhinoviruses can produce experimental infection only in chimpanzees. Related rhinoviruses have been isolated from cattle and horses, but their significance in human infection is not known.

Rhinoviruses can be grown in tissue cultures of human or simian origin with cytopathic changes, if good oxygenation (achieved by rolling), low pH (around 7) and low temperature (33°C) are provided. Depending upon growth in tissue culture, rhinoviruses are classified into H and M strains. H strains grow only in human cells while M strains grow equally well in human and monkey cells.

Epidemiology: Common cold is an infectious disease transmitted by droplets. The incubation period is about two days. The duration of virus shedding is not known, though it is unlikely to be prolonged. Contrary to popular belief, there is no direct relation between inclement weather and common cold. The multiplicity of serotypes makes vaccination impossible. Moreover, common cold is a syndrome produced not only by rhinoviruses, but also by a variety of other groups such as respiratory syncytial, corona, coxsackie, echo, adeno, influenza and parainfluenza viruses. Hope of specific control therefore lies in the development of antiviral chemotherapy.

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55 Orthomyxovirus

The name Myxovirus was proposed originally for a group of enveloped RNA viruses characterised by their ability to adsorb on to mucoprotein receptors on erythrocytes, causing *haemagglutination*. The name referred to the affinity of the viruses to mucins (from *myxa*, meaning mucus.) Included in this group were influenza, mumps, Newcastle disease and parainfluenza viruses. The subsequent recognition of important differences between influenza viruses and the other viruses in this group led to their being reclassified into two separate families — Orthomyxoviridae consisting of the influenza viruses and Paramyxoviridae consisting of the Newcastle disease virus, mumpsvirus, parainfluenzaviruses, measles and respiratory syncytial viruses. Table 55.1 lists the important differences between orthomyxovirus and paramyxovirus.

INFLUENZA

Influenza is an acute infectious disease of the respiratory tract which occurs in sporadic, epidemic and pandemic forms. The name 'influenza' is said to have been given by Italians during the epidemic of 1358, which they ascribed to

TABLE 55.1

Distinguishing features of orthomyxovirus and paramyxovirus

Property	Orthomyxovirus	Paramyxovirus
Size of virion	80-120 nm	100-300 nm
Shape	Spherical; filaments *in fresh isolates*	Pleomorphic
Genome	Segmented: eight pieces of RNA	Single linear molecule of RNA
Diameter of nucleocapsid	9 nm	18 nm
Site of synthesis of ribonucleoprotein	Nucleus	Cytoplasm
Genetic recombination	Common	Absent
DNA-dependent RNA synthesis	Required for multiplication	Not required
Effect of Actinomycin D	Inhibits multiplication	Does not inhibit
Antigenic stability	Variable	Stable
Haemolysin	Absent	Present

the malevolent influence of the heavenly bodies or of inclement weather. The modern history of the disease may be considered to date from the pandemic of 1889–1890, during which Pfeiffer isolated *Haemophilus influenzae* and claimed it to be the causative agent. The most severe pandemic occurred in 1918–1919, when it was shown that Pfeiffer's bacillus was not the primary cause of the disease, though it may act as a secondary invader. The isolation of the influenza virus in 1933 by Smith, Andrewes and Laidlaw was a milestone in the development of medical virology. They reproduced the disease in ferrets by intranasal inoculation with bacteria free filtrates of nasopharyngeal secretions from patients. Burnet (1935) developed chick embryo techniques for propagation of the virus.

A notable advance was the independent discovery by Hirst, and by McClelland and Hare (1941) that influenza viruses agglutinate fowl erythrocytes. The property of haemagglutination was found to be a common feature of many other viruses.

Francis and Magill (1940) independently isolated a serotype of influenza virus which was unrelated to the strains known till then. This was designated influenza virus type B, to distinguish it from the original serotype, which was named type A. Taylor (1949) isolated the third serotype of influenza virus, type C. The classification of influenza viruses into the three serotypes, A, B and C, is based on the antigenic nature of the 'internal' or ribonucleoprotein antigen.

Influenza occurs also in animals and birds in nature. Indeed, the avian influenza virus was demonstrated as early as in 1901, when Centanni and Avonuzzi showed that fowl plague was a virus disease. But, as fowl plague is a septicaemia so different clinically from human influenza, the association between the two remained unknown till 1955, when Schaefer demonstrated that the fowl plague virus was antigenically related to the influenza virus. Shope (1931) isolated the swine influenza virus. Not only did the swine disease resemble human influenza clinically, but there was also epidemiological association between the two. It was widely held that the virus spread to swine from man at the time of the 1918 pandemic. Influenza viruses have also been isolated from horses, whales and seals. Birds appear to be an important reservoir of influenza virus and natural infection has been identified in several avian species. The cloaca of healthy wild birds was found to give greater isolations of influenza viruses than the respiratory tract. All isolates from nonhuman hosts belong to type A. Influenza virus types B and C appear to be exclusively human viruses and natural infection with them has not been identified in animals or birds. However, in 1983, influenza virus type C isolations have been reported from pigs in China and antibodies demonstrated in them. Ordinarily, nonhuman influenza viruses do not cause human infection. But they may play an important role in the emergence of pandemic influenza.

Influenza viruses

Morphology: The influenza virus is typically spherical, with a diameter of 80 – 120 nm, but pleomorphism is common. Filamentous forms, upto several microns in length and readily visible under the dark ground microscope, are frequent in freshly isolated strains.

The virus core consists of ribonucleoprotein in helical symmetry. The single-stranded RNA genome is segmented and exists as eight pieces. The nucleocapsid is surrounded by an envelope. It has an inner 'membrane protein' layer and an outer lipid layer. The membrane protein is also known as the 'matrix' or 'M protein'. The protein part of the envelope is virus coded, but the lipid layer is derived from the modified host cell membrane, during the process of replication by budding. Attached to the lipid layer of the envelope are two types of peplomers — haemagglutinin spikes which are triangular in cross section and the mushroom shaped neuraminidase peplomers which are less numerous (Fig. 55.1).

Resistance: The virus is inactivated by heating at 50°C for 30 minutes. It remains viable at 0–4° C

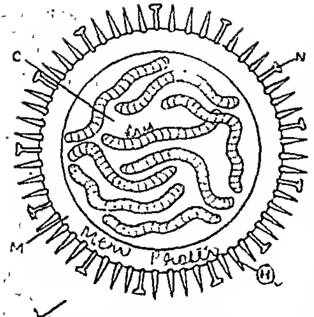

Fig 55.1 Diagramatic representation of influenza virus as revealed by the electron microscope (H) haemagglutinin N. neuraminidase C core containing eight strands of RNA M membrane protein

for about a week. Infectivity is lost rapidly at -20°C. It can be preserved for years at -70°C or by freeze drying. The virus survives slow drying and may remain viable on fomites such as blankets for about two weeks. Ether, formaldehyde, phenol, salts of heavy metals and many other chemical disinfectants destroy infectivity. Iodine is particularly effective.

Haemagglutinating, enzymic and complement fixing activities of the virus are more stable than infectivity.

Haemagglutination: Haemagglutination is an important characteristic of influenza viruses. When mixed with a suspension of fowl erythrocytes, the virus is adsorbed on to the mucoprotein receptors on the cell surface. The virus links together adjacent cells producing haemagglutination. The haemagglutinin peplomers on the viral surface are responsible for this activity. Haemagglutination is followed after a time by the detachment of the virus from the cell surface, reversing the haemagglutination. This process is known as 'elution' and is caused by the enzyme neuraminidase present on the viral surface. The enzyme acts on the cell receptor, destroying it by splitting off N-acetyl neuraminic acid from it.

Virus particles which have eluted from red cells are still capable of agglutinating fresh red cells, but red cells that have been acted on by the virus are not susceptible to agglutination by the same strain of virus. Such red cells may, however, be agglutinated by other serotypes of virus. This inability of these red cells to be reagglutinated by the same virus is due to the destruction of the specific cell receptors by the initial treatment with the virus. Influenza viruses differ in their ability to destroy cell receptors. Myxoviruses can be arranged in a series in which the treatment of red cells with any one virus removes the receptors for that virus and the preceding viruses, but not for the viruses later in the series. This is called the 'receptor gradient'. For myxoviruses in general, the gradient is mumps, Newcastle disease virus and influenza, in that order. MNI

Neuraminidase is an isoenzyme and different serotypes of influenza virus possess enzymes that vary in their characteristics such as antigenic structure, temperature optima and heat stability. Neuraminidases are also present in bacteria and in cells of higher organisms. Culture filtrates of *V. cholerae* are rich in neuraminidase activity and red cells pretreated with them are resistant to haemagglutination by influenza viruses. The culture filtrate was, therefore, called the receptor destroying enzyme (RDE) of *V. cholerae*.

Haemagglutination takes place at a wide range of temperature, from 0°C to 37°C. Elution, on the other hand, being an enzymic reaction does not occur at 0°C, but is rapid at 37°C. Influenza viruses vary in their ability to agglutinate red cells of different species. In general, influenza virus types A and B agglutinate erythrocytes of fowl, human, guinea pig and some other species. Influenza virus type C agglutinates red cells of fowl only, at 4°C.

Haemagglutination provides a convenient method for the detection and titration of influenza virus in egg and other culture fluids. The highest dilution of the virus suspension that

produces agglutination of a fixed quantity of cells is known as its haemagglutination titre. Haemagglutinin is more resistant to physical and chemical agents than infectivity. Therefore, haemagglutination can be used for the titration of inactivated influenza virus also, as for example, in the standardisation of killed influenza virus vaccines. Haemagglutination is inhibited by the specific antibody. Haemagglutination inhibition, therefore, offers a convenient method for the detection and quantitation of antibody to the virus. A disadvantage of this serological technique is the frequent presence in sera of certain substances that cause nonspecific inhibition of haemagglutination. Different kinds of nonspecific inhibitors have been identified in sera and have been given names such as *alpha* (Francis), *beta* (Chu), and *gamma* (Shimojo) inhibitors. They are mostly glycoproteins. A variety of techniques have been introduced for inactivating them without affecting the antibody content of sera. These include treatment with RDE, trypsin, potassium periodate, kaolin and CO_2. No single method has been found effective in destroying completely inhibitors to all types of viruses from all kinds of sera. Virus strains vary in their susceptibility to nonspecific inhibitors. When available, the use of a strain insusceptible to such inhibitors would enhance the value of haemagglutination inhibition tests.

Haemagglutination and elution can be used for purifying and concentrating influenza viruses. The plasma membranes of cells in which the virus is multiplying contain the haemagglutinin. Therefore, red cells are adsorbed on to the surface of such cells. This is the basis of haemadsorption, a technique by which the growth of the influenza virus in cell cultures can be identified.

Antigenic structure: The antigens of the influenza virus can be classified as the 'internal antigens' and the 'surface antigens'. The internal antigen is the ribonucleoprotein and is hence called the RNP antigen. Because it is found free in infected tissues and occurs in the supernatant when the virus containing fluid is centrifuged, it was also called the 'soluble' (S) antigen. The RNP antigen can be demonstrated by complement fixation and immunoprecipitation tests. It is type specific and based on its nature, influenza viruses are classified into types A, B and C. The RNP antigens of types A, B and C are distinct, but all strains of any one type possess the same antigen. The RNP antigen is stable and does not exhibit any significant antigenic variation. The antibody develops following infection, but not ordinarily in persons immunised with killed vaccines.

The 'membrane protein' antigen is type specific. The envelope lipid antigen is host specific and is determined by the species in which virus replication takes place.

The term 'viral' or V antigen was used formerly to describe the surface antigen of the influenza virus. Antibodies to the V antigen were estimated by complement fixation. The V antigen is actually composed of at least two virus coded proteins, the haemagglutinin and the neuraminidase. The two proteins have been isolated and purified.

The haemagglutinin is a glycoprotein composed of two polypeptides — HA 1 and HA 2. It is responsible for haemagglutination and haemadsorption. It enables the virus to adsorb to mucoprotein receptors on red cells as well as on respiratory epithelial cells. Antihaemagglutinin antibodies are produced following infection and immunisation. This antibody is protective by preventing adsorption of virus to cells. The haemagglutinin is a strain specific antigen and is capable of great variation.

The neuraminidase is a glycoprotein enzyme which destroys cell receptors by hydrolytic cleavage. The antineuraminidase antibody is formed following infection and immunisation. It is not as effective in protection as the antihaemagglutinin antibody. It does not prevent the adsorption of virus on to cells, but can inhibit the release of progeny virions and may thus contribute to limiting the infection. The neuraminidase is a strain specific antigen and exhibits variation.

Antigenic variation: A unique feature of the

influenza virus is its ability to undergo antigenic variation. This is of great importance in the epidemiology of the disease. Antigenic variability is highest in the influenza virus type A and less in type B, while it has not been demonstrated in type C.

The internal RNP antigen is stable, but both the surface antigens, haemagglutinin and neuraminidase undergo independent antigenic variations. Depending on its degree, antigenic variation has been classified as 'antigenic drift' and 'antigenic shift'. The gradual sequential change in antigenic structure occurring regularly at frequent intervals is known as antigenic drift. Here, the new antigens, though different from the previous antigens, are yet related to them, so that they react with antisera to the predecessor virus strains, at least to a limited extent. Antigenic drift is due to mutation and selection, the process being influenced by the presence of antibodies to the predecessor strains in the host population. The antigenic drift accounts for the periodical epidemics of influenza.

Antigenic shift, on the other hand, is an abrupt, drastic, discontinuous variation in the antigenic structure, resulting in a novel virus strain unrelated antigenically to predecessor strains. Such changes may involve the haemagglutinin, the neuraminidase or both. Antibodies to predecessor viruses do not neutralise the new variants and they can, therefore, spread widely in the population causing major epidemics or pandemics. The changes involved in the antigenic shift are so extensive that they are unlikely to be the result of mutation.

Antigenic classification: Influenza virus type A strains can be classified into subtypes based on variations in their surface antigens. Originally only variations in the haemagglutinin were studied, and subtyping depended only on antigenic shifts occurring in the haemagglutinin. The earliest isolates (WS, PR8 and related strains) were designated A0. In 1946, the haemagglutinin underwent a major change and these strains (CAM, FM1 and others) were named A1 or A' (A prime). In 1957, new pandemic strains originated in Asia. These were called the A2 (Asian) strains. The next major change occurred in 1968 with the emergence of the A2 (Hong Kong) subtype. Within each of these subtypes, the strains have shown a gradual antigenic drift.

With the recognition that viral neuraminidase also undergoes independent antigenic variation, a new system of classification was proposed by the WHO in 1971, which took into account the nature of both the surface antigens. According to this, the haemagglutinins of A0, A1, A2 (Asian) and A2 (Hong Kong) are named H0, H1, H2 and H3, respectively. The neuraminidases of human influenza type A viruses belong to two subtypes — the subtype N1 occurring in A0 and A1 strains, and subtype N2 in A2 (Asian) and A2 (Hong Kong) strains. The complete designation of a strain will include the type, place of origin, serial number and year of isolation followed by the antigenic subtypes of the haemagglutinin and neuraminidase in parenthesis, for example A/Hong Kong/1/68 (H3 N2).

TABLE 55.2

Antigenic subtypes of influenza virus type A

Old designation	*Subtype*	*Reference strains*
A0	H0N1	A/PR/8/34
A1	H1N1	A/FM/1/47
A2	H2N2	A/Singapore/1/57
A2(Hong Kong)	H3N2	A/Hong Kong/1/68

TABLE 55.3

Representative influenza viruses of animals and birds

Host	Date of isolation	Place of isolation	Antigenic structure	
Swine	1930	USA	Hsw1	N1
	1970	Taiwan	H3	N2
Horse	1957	Prague	Heq1	Neq1
	1963	Miami	Heq2	Neq2
Chicken	1927	Indonesia	Hav1	Neq1
	1934	Rostock	Hav1	N1
	1949	Germany	Hav2	Neq1
	1959	Scotland	Hav5	N1
Duck	1956	England	Hav3	Nav1
	1956	Czechoslovakia	Hav4	Nav1
	1963	Ukraine	Hav7	Neq2
	1966	Italy	Hav2	N2
	1968	Germany	Hav6	N1
	1973	Germany	H2	Nav2
	1974	USA	Hav3	N6
	1976	Alberta	Hav10	Nav5
Tern	1961	Canada	Hav6	Neq2
Turkey	1963	England	Hav1	Nav3
	1965	Massachusetts	Hav6	N2
	1966	Wisconsin	Hav9	N2
	1968	Ontario	Hav8	Nav4
Quail	1965	Italy	Hav2	Neq2
Shearwater	1971	Queensland	Hav6	Nav5
Whale	1978	Pacific Ocean	H_{sw}	N1
Seal	1980	N.E. Coast of America	Hav1	Neq.1

Table 55.2 shows the antigenic subtypes of type A virus.

This scheme of classification was applicable to the influenza viruses of nonhuman hosts also. The suffix 'sw', 'eq', or 'av' after 'H' or 'N' referred to their origin from pigs, horses or birds (Table 55.3)

With their detailed antigenic and biochemical characterisation it was realised that several H and N antigens of the human and nonhuman influenzavirus subtypes were identical. Therefore, the WHO, in 1980, proposed a new designation which abolished the suffixes 'sw', 'eq' and 'av' and gave serial numbers for the H and N antigens

irrespective of their host of origin. Thus H3, Heq 2, and Hav 7 were all designated H3 because they were shown to be identical (Table 55.4). However, till these designations become widely accepted, the old nomenclature also continues to be in use.

Influenza virus type B also exhibits antigenic variation, but the changes have not been marked enough for the subtypes to be delineated. Type C virus has not undergone any antigenic variation.

P-Q-R variation: Influenza virus strains belonging to the same subtype—even strains isolated during the course of a single outbreak — may behave differently in neutralisation tests with antisera. Van der Veen and Mulder called this the *P-Q-R variation*. Strains in the P phase were neutralised by the homologous antiserum in high titre and by heterologous antiserum in low titre. Strains in the Q phase were neutralised poorly by either homologous or heterologous sera. R phase strains were neutralised by both homologous and heterologous sera in high titre. The P-Q-R variation appears to be due to the rearrangement of antigenic determinants on the surface of the virion. In the P phase, the dominant antigen is on the surface so that it reacts with the specific antibody with high avidity while in the Q phase the dominant antigen is apparently buried under the surface and hence inaccessible to the antibody. The P-O-R phases are interconvertible by passaging the virus in the presence of the appropriate antiserum.

O-D variation: Burnet and Bull (1943) observed that influenza virus type A underwent certain changes when serially passaged in eggs. They called this the O-D variation. The fresh isolate was said to be in the 'Original' (O) phase and the passaged virus in the 'Derived' (D) phase. The O phase virus grew well in the amniotic cavity of chick embryos, but only poorly or not at all in the allantoic cavity. It agglutinated mammalian erythrocytes (guinea pig and human) to a high titre

TABLE 55.4

Current designations of H and N antigens of influenza viruses

Haemagglutinin Subtypes		Neuraminidase Subtypes	
Previous designation	*Current* designation (1980)	*Previous* designation	*Current* designation (1980)
H0,H1,Hsw1	H1	N1	N1
H2	H2	N2	N2
H3,Heq2,Hav7	H3	Nav2,Nav3	N3
Hav4	H4	Nav4	N4
Hav5	H5	Nav5	N5
Hav6	H6	Nav1	N6
Heq1,Hav1	H7	Neq1	N7
Hav8	H8	Neq2	N8
Hav9	H9	Nav6	N9
Hav2	H10		
Hav3	H11		
Hav10	H12		
Nil as first isolated after 1980	H13		

and fowl erythrocytes only weakly or not at all. Filamentous forms were common. It was infectious for man. The D phase virus, on the other hand, grew equally well in the amniotic and allantoic cavities, agglutinated fowl erythrocytes as well as, or better than, mammalian cells, showed no filamentous forms and was relatively avirulent for man. The O-D variation was considered to result from mutation.

Host range: Human influenza virus can cause experimental infection in a number of animal species. In most, the infection is asymptomatic, though virus shedding occurs from the respiratory tract for a few days. Intranasal inoculation in ferrets produces an acute respiratory disease. This was the manner in which the influenza virus was first isolated. Strains vary considerably in virulence to ferrets, some producing severe febrile disease and others only asymptomatic infection. The virus can be 'adapted' by serial intranasal passage in mice to produce fatal pulmonary infection. Neurotropic mutants have been isolated which regularly produce fatal encephalitis after intracerebral inoculation in mice.

The virus grows well in the amniotic cavity of chick embryos. After a few egg passages, the virus grows well in the allantoic cavity also, except for type C virus which does not generally grow in the allantoic cavity. The influenza virus does not damage chick embryos, which may hatch out normally. Virus growth is detected by the appearance of haemagglutinin in the allantoic and amniotic fluids.

The virus grows in monkey kidney cell cultures. Cytopathic effects are not prominent and virus growth is detected by haemadsorption or demonstration of haemagglutinin in the culture fluid.

When passaged serially in eggs, using as inocula undiluted infected allantoic fluid, the progeny virus will show high haemagglutinin titre, but low infectivity. This has been called the Von Magnus phenomenon and is due to the formation of incomplete virus particles lacking nucleic acid.

Pathogenesis: The route of entry is the respiratory tract. In experimental infection in volunteers, very small doses (approximately three viable particles) can initiate infection when given as aerosols. Larger doses are required when infection is by intranasal instillation. The viral neuraminidase facilitates infection by reducing the viscosity of the mucus film lining the respiratory tract and exposing the cell surface receptors for virus adsorption. The ciliated cells of the respiratory tract are the main sites of virus infection. These cells are damaged and shed, laying bare the basal cells in the trachea and bronchi. This renders the respiratory tract highly vulnerable to bacterial invasion. Viral pneumonia, seen only in the more severe cases, is associated with hyperaemia and thickening of alveolar walls, interstitial infiltration with leucocytes, capillary thrombosis and leucocytic exudation. In some cases a hyaline membrane is formed, occupying the alveolar ducts and alveoli. In the late stages, there is infiltration with macrophages which engulf and remove desquamated alveolar cells.

The disease is ordinarily confined to the respiratory tract. Very rarely, the virus had been isolated from the spleen, liver, kidneys and other organs during the 1957 pandemic.

Clinical features: The incubation period is 1–3 days. The disease varies in severity from a mild coryza to fulminating and rapidly fatal pneumonia. Most infections are subclinical. In the typical clinical disease, the onset is abrupt, with fever, headache and generalised myalgia. Respiratory symptoms are prominent and severe prostration is common. Abdominal pain and vomiting may occur, especially in type B infection in children, which may even present as an acute abdominal emergency. The uncomplicated disease resolves within 2–7 days.

The most important complication is pneumonia, which is mainly due to bacterial superinfection or, rarely, caused by the virus itself. Cardiac complications, such as congestive failure or myocarditis and neurological involvement, such as encephalitis may occur rarely.

Influenza, particularly infection with type B, has been associated with Reye's syndrome. It specially affects young children and is characterised by acute degenerative changes in the brain, liver and kidneys. The possibility of congenital defects if infection occurs in early pregnancy has been mooted.

Laboratory diagnosis: 1. Demonstration of virus antigen: Rapid diagnosis of influenza may be made by demonstration of the virus antigen on the surface of the nasopharyngeal cells by immunofluorescence. Smears of nasal swabs and nasopharyngeal secretions or centrifuged deposit of throat garglings are prepared on slides and treated with fluorescent conjugated influenza virus antiserum and examined under the ultraviolet microscope. The cells will be found to fluoresce due to the presence of viral antigens on the cell surface.

2. Isolation of the virus: Virus isolation is readily obtained from the patients during the first two or three days of the illness, but usually not in the later stages. Throat garglings are collected using broth saline or other suitable buffered salt solution. If the specimen is not processed immediately, it should be stored at 4°C, or if the delay is long, at −70°C. The specimen should be treated with antibiotics to destroy bacteria. Isolation may be attempted in eggs or in monkey kidney cell culture.

The material is inoculated into the amniotic cavity of 11–13 day old eggs, using at least six eggs per specimen. After incubation at 35°C for three days, the eggs are chilled and the amniotic and allantoic fluids harvested separately. The fluids are tested for haemagglutination using guinea pig and fowl cells in parallel, at room temperature and at 4°C. Some strains of the influenza virus type A agglutinate only guinea pig cells on initial isolation. The type B virus agglutinates both cells, while type C strains agglutinate only fowl cells at 4°C. If no haemagglutination is seen, a blind passage of the chick embryo lung and liver homogenate into another set of eggs may sometimes lead to recovery of the virus. The isolate is identified and typed by complement fixation test with antisera to types A, B and C. Subtype identification is made by haemagglutination inhibition test. Some of the recent type A strains can be isolated by direct allantoic inoculation of the clinical specimen into 9–11 day old eggs. But type B and C viruses will be missed if only allantoic inoculation is used.

Inoculation into monkey kidney cell cultures is nearly as efficient as egg inoculation for isolation of type A strains, and slightly more sensitive for type B strains. Inoculated cell cultures are incubated at 33°C in roller drums. Virus growth can be identified by testing the culture fluid for haemagglutination or by haemadsorption. Rapid results can be obtained by demonstrating virus antigen in infected cell cultures by immunofluorescence.

Serology: The complement fixation and haemagglutination inhibition tests are routinely employed for the serological diagnosis of influenza. It is essential to examine paired sera in parallel, to demonstrate rise in titre of antibodies.

Complement fixation tests with the RNP antigen of influenza virus types A, B and C are very useful as the antibodies are formed during infection, but not following immunisation with inactivated vaccines. Complement fixation can also be done using V antigens for the demonstration of strain specific antibodies.

Haemagglutination inhibition is a convenient and sensitive test for the serological diagnosis of influenza. But it has some disadvantages. As the antihaemagglutinin antibodies are subtype specific, it is necessary to use as antigen the strain currently causing infection. The major drawback is the frequent presence in the sera of nonspecific inhibitors of haemagglutination. The sera, suitably treated for the removal of nonspecific inhibitors, are diluted serially in perspex haemagglutination plates and the influenza virus suspension containing 4 HA units added to each cup. Fowl red cells are then added. The highest dilution of serum that inhibits haemagglutination is its HI titre.

It is possible to estimate the neuraminidase antibody by enzyme neutralisation tests. But these are too cumbersome for routine use.

Radial immunodiffusion tests in agarose gel have been described for the identification of antibodies to the RNP antigen, haemagglutinin and neuraminidase. But these are more useful as screening tests than for routine diagnosis.

Immunity: An attack of influenza confers protection effective for about one or two years. The apparent short duration of immunity is due to the antigenic variation that the virus undergoes frequently. Following infection and immunisation, circulating antibodies are formed against the various untigens of the virus. But it is the local concentration of antihaemagglutinin and to a smaller extent, of antineuraminidase antibodies (mainly IgA) in the respiratory tract that is more relevant in protection

When an individual experiences repeated infections with different antigenic variants of influenza virus type A, he responds by forming antibodies not only against each infecting strain, but also against the strain that he had come into contact with first. The dominant antibody response will be against the strain that caused the earliest infection. This phenomenon has been called the doctrine of 'original antigenic sin'.

Influenza virus infection induces cell mediated immunity also, but its role in protection has not been clarified.

Epidemiology: Influenza occurs sporadically, as epidemics or in pandemic form. The source of infection is an infected individual. The virus is shed in the respiratory secretions shortly before the onset of illness and for 3–4 days thereafter. Subclinical infections are common. Influenza virus type C is endemic throughout the world and causes very mild or inapparent infections. Type B strains cause sporadic as well as epidemic influenza, while type A strains can cause pandemics as well. Sporadic influenza is of little public health importance as it is a mild self-limited condition. Epidemic influenza is important in the temperate regions where it strikes during the winter months, producing considerable mortality in the aged and in those with cardiopulmonary diseases. In the tropics, epidemic influenza does not exhibit a winter prevalence, though it tends to occur frequently in the monsoon season.

What makes influenza an important and challenging disease is its propensity for the production of pandemics. It is for this reason that worldwide surveillance is maintained on influenza, under the auspices of the WHO. Influenza pandemics have been recorded at irregular intervals from 1173. Pandemics of modern times date from 1889. The most severe pandemic in human history occurred in 1918–1919, during which over 20 million people perished. An unusual feature of this pandemic was the very high rate of mortality among young adults. The next pandemic ocurred in 1957 when the Asian strain originated in China and spread throughout the world within a short period. But, though it caused widespread morbidity, the mortality rate was low. The Hong Kong strain originating in 1968 also caused a pandemic, but it was much less severe.

In 1977, epidemic influenza appeared in China and then in Russia (hence called the 'red flu' facetiously). The disease was mainly confined to the under-20 age group. The isolate was identified as H1N1 virus, antigenically very close to the strains prevalent in 1950. This H1N1 virus has spread through most of the world, and, with H3N2 virus, currently cause human influenza.

The reason why the virus is able to cause epidemics and pandemics is its ability to undergo antigenic variations. Antigenic drift, resulting from mutation and selection, is responsible for the epidemics. It has been shown experimentally that passaging the virus in the presence of antiserum leads to the appearance of such mutants. Pandemics are caused by a virus strain that has undergone antigenic shift. The variation in such instances is so marked and involves different polypeptides simultaneously, that mutation cannot explain it. It is now believed that pandemic strains originate from some animal or avian reser-

voir, either spreading to man directly by host range mutation, or as a result of recombination between human and nonhuman strains. There is considerable evidence to support the view that pandemic strains represent such hybrids. Hybrids can be produced by growing together in eggs, human and nonhuman strains. Recombinants can also be obtained from experimental animals exposed to mixed infection. The observation that some human and nonhuman strains share the same haemagglutinin or neuraminidase antigens strengthens this hypothesis.

Recent events have further confounded the already confused picture of the epidemiology of influenza. The reasons for the sudden reappearance of the Hsw1N1 virus in 1976 and its rapid disappearance remain mysterious. Similarly, it is not known how or why the H1N1 virus reappeared in 1977 after an absence of two or three decades. It has been postulated that old strains that disappear from human communities may persist for prolonged periods in the natural deep freeze conditions existing in the Antarctic and South Pacific, probably in a biological cycle involving the zooplanktons so abundant there. The virus may possibly be deposited there by bird droppings. The recent isolation of 'old' influenzavirus strains from whales in the South Pacific lends credence to this hypothesis. Another possibility is that 'old' viruses may persist as a latent infection in man (or in human lung parasites) and re-emerge after several years, reminiscent of the swine lungworm-earthworm cycle postulated by Shope for swine influenza virus.

A unique feature of influenza epidemiology was that once an antigenic variant emerged, it displaced completely the pre-existing strain. Thus when A1 (H1N1) strains arose in 1946–47, they became the only viruses causing human disease, and the previous AO (HON1) strains disappeared completely. The A1 strains were displaced by Asian (H2N2) strains in 1957, and they, in turn, by the A2 Hong Kong (H3N2) strains in 1968. But this rule has not been observed in recent years. Even after the abortive reappearance of Hsw1N1 strains in 1976 and the re-emergence and wide spread of H1N1 strain in 1977, the A2 Hong Kong H3N2, strains continue to be prevalent. The reason for this coexistence is not known.

There is considerable evidence to suggest that there occurs an orderly recycling of the virus subtypes. Sero-epidemiological studies indicate that the pandemic of 1889 was caused by a virus resembling the A2 Asian (H2 N2) subtype and that the Hong Kong (H3N2) subtype was prevalent after 1900. This was followed by the AO (HON1) subtype in the 1930's and the A1 (H1N1) subtype in 1946–47. The cycle started again with the reappearance of the A2 Asian (H2N2) subtype in 1957. Table 55.5 lists the sequence of appearance of these various subtypes.

Prophylaxis: Influenza vaccines have been in use for over three decades. The original vaccines consisted of the virus grown in the allantoic cavity of eggs, partially purified, and inactivated with formalin. Due to the presence of egg protein in it, this vaccine may cause reactions in allergic individuals. The whole virus vaccine induces fever and local pain. 'Subunit' vaccines have been introduced to minimise toxic reactions. The purified virus is disrupted by treatment with ether or desoxycholate so that the vaccine contains the immunogenic haemagglutinin and neuraminidase subunits.

The major difficulty in the immunoprophylaxis of influenza is the frequent change in the antigenic make up of the virus. Vaccines cannot be made in bulk and stockpiled, as the appearance of a new variant will make the old vaccine obsolete. In the cold countries, where it is necessary to provide protection to old persons and other high risk individuals, the practice is to immunise them with a vaccine containing the latest strains of type A and B viruses

The most important indication for immunoprophylaxis is when a pandemic is threatened by a new virus. Here, the time taken for the manufacture of the vaccine with the new variant is crucial, as the virus is likely to spread fast and infect

TABLE 55.5

Genealogy of Influenzavirus A subtypes. (Data before 1933 based on serological and epidemiological evidence)

Antigenic structure	Date of origin and duration	Remarks
H2N2	1889-1890 ↓ 1900	Pandemic believed to have been caused by 'A2-like' virus. Origin unknown. Progressive antigenic drift of H2N2 strains.
		ANTIGENIC SHIFT
H3N2	↓ 1918	'Hong Kong-like' virus believed to have appeared around 1900. Origin unknown. Progressive antigenic drift till 1918.
		ANTIGENIC SHIFT
$H_{sw}1N1$	1918-1919 ↓	Severe pandemic caused by 'Asw like' virus. Origin unknown. Antigenic drift of $H_{sw}1N1$ strains.
H0N1	1933 ↓ 1946	Discovery of human influenza virus (WS strain, H0N1). Antigenic drift till 1946-47.
H1N1	1946-47 ↓	Emergence of A1 strains by mutation from H0N1 Antigenic drift till 1957.
		ANTIGENIC SHIFT
H2N2	1957 ↓	Pandemic caused by 'A2 (Asian)' virus. Origin unknown, but possibility of spread from 'animals' considered. Antigenic drift till 1968.
		ANTIGENIC SHIFT
H3N2	1968 ↓ TO THE PRESENT	Emergence and spread of 'A2 (Hong Kong)' virus. Origin unknown, but probably by recombination, from animal or avian influenza virus. Antigenic drift continuing.
$H_{sw}1N1$	1976	Reappearance of 'Asw-like' virus in USA with limited local spread only. Origin Unknown.
H1N1	1977 TO THE PRESENT	Reappearance of H1N1 virus (resembling 1950 strains), first in China and Russia, and spreading worldwide. Origin unknown.

whole populations before the vaccine becomes available. Moreover, most fresh isolates do not grow well in eggs till they are passaged serially. To overcome these hurdles, the recombinant vaccine has been introduced. A recombinant is produced which possesses the growth characters of old established strains and carries the surface antigens of the new variant. The recombinant will grow well in eggs facilitating vaccine manufacture.

While killed vaccines induce the formation of circulating antibodies, they do not lead to any local protection in the respiratory tract. The level of antibodies on the respiratory mucosa is only a fraction of the serum level. It is in order to afford specific local immunisation that live vaccines have been employed. The earliest live vaccine was the virus attenuated by repeated egg passage. It was administered by intranasal instillation. But it sometimes gave rise to clinical disease, especially in children. Another approach to live vaccine is the use of temperature sensitive mutants. Mutants can be readily isolated which are able to grow at the lower temperature of the nasopharyngeal mucosa (32°C–34°C), but not in the lungs at 37°C. Such *ts* mutants are avirulent. Recombinant live vaccines may be obtained by hybridisation between *ts* mutants of established strains and a new antigenic variant.

Chemoprophylaxis has been reported to be successful with the antiviral drug amantadine hydrochloride, which inhibits adsorption of influenza type A virus to cells.

Treatment: Amantadine hydrochloride has been found to be of some value in the treatment of influenza. It reduces the average duration of the disease and causes symptomatic improvement, though virus shedding and antibody response are not affected.

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56 Paramyxoviruses

Paramyxoviruses resemble orthomyxoviruses in their morphology, but are larger and more pleomorphic. They range from 100–300 nm in size, with occasional filaments and giant forms upto 800 nm. The helical nucleocapsid has a diameter of 18 nm (much wider than in orthomyxovirus) and contains a molecule of single stranded RNA (MW 7 million daltons), arranged as an unsegmented filament 1μ long.

Paramyxoviruses possess haemagglutinins and neuraminidases as do orthomyxoviruses. In addition, they also have haemolysins. They have the ability to cause cell fusion leading to the formation of giant cells and syncytia. They are antigenically stable. Though there is no common group specific antigen, the different paramyxoviruses exhibit serological cross reactions.

The family paramyxoviridae consists of three genera. The genus *Paramyxovirus* includes the 'authentic' paramyxoviruses — Newcastle disease, mumps and parainfluenza viruses. Measles virus and the related virus of canine distemper and bovine rinderpest belong to the genus *Morbillivirus* They possess haemagglutinins but no neuraminidase. The respiratory syncytial virus and some related viruses of animals have been placed in the genus *Pneumovirus* These possess neither haemagglutinins nor neuraminidase.

Mumps

Hippocrates in the fifth century BC described the condition first. The word 'mumps' is probably derived from the British verb 'to mump' which means 'to grimace or grin'. It was known as epidemic parotitis till it was shown that other viruses such as the coxsackie, parainfluenza, lymphocytic choriomeningitis, influenza A, echo and possibly herpes simplex and varicella-zoster viruses, also cause parotid swelling. Hamilton in late 18th century associated CNS involvement with the disease. Johnson and Goodpasture (1934) first convincingly proved its association with a virus. Habel in 1945 cultivated the causative virus in embryonated eggs. In 1955, Henle and Deinhardt grew it in tissue culture.

Properties of the virus: The virus varies in size from 100 to 600 nm and is markedly pleomorphic. The virions are roughly spherical composed of an outer membrane enclosing an inner helical structure. The outer membrane is covered by projections extending 12–15 nm from the virion surface. The coiled inner structure is the nucleocapsid and is an RNA complex. Filamentous and bizarre forms of the virus are frequently found.

The virus contains five major structural proteins: The prominent nucleocapsid associated protein (NP), a polymerase protein (P), a membrane or matrix protein (M) and two glycoproteins — a haemagglutinin-neuraminidase (HN) and, a fusion protein (P), which is also a haemolysin. The virion also probably contains a high MW, L, protein.

The virus agglutinates the erythrocytes of fowl, human and other species. Haemagglutination is followed by haemolysis and elution at 37°C.

The virus can be grown, on primary isolation, in the yolk sac or amniotic cavity of chick embryos. As growth is slow, 6–8 day old embryos are

inoculated and incubated for five days at 35°C. After adaptation to eggs, most strains grow well in the allantoic cavity.

The primary isolation of the virus can also be obtained in human or monkey kidney cell cultures. The cytopathic effect is weak and consists of syncytium formation and the presence of acidophilic cytoplasmic inclusions. Growth is best identified by haemadsorption.

The virus is easily inactivated at room temperature or by exposure to formaldehyde, ether or ultraviolet light. It can be preserved at –70°C or by lyophilisation.

The virus is antigenically stable. Two complement fixing antigens can be recognised, as in the influenza viruses, the 'soluble' (S) antigen and the 'viral' (V) antigen.

Clinical features: Infection is acquired by inhalation and probably also through the conjunctiva. Mumps virus may also be transmitted by fomites. The incubation period is long, about 10–21 days. The virus is present in the saliva of infected individuals for several days before the onset of clinical disease and shed for nine days into the course of symptoms. A prodromal period of malaise precedes the swelling of one or both the parotid glands and of other salivary glands. Virus is actively shed in the saliva for as long as seven days before the onset of clinical disease. The presence of virus in saliva is not dependent on the development of clinical parotitis. Mumps patients are capable of spreading the virus by the respiratory route for 7–10 days. The virus may reach the parotid gland either directly from the mouth by way of the Stenson's duct or by haematogenous spread after initial multiplication in the respiratory tract. A cell associated viraemia may be another important mode of virus transmission. Viraemia is responsible for the involvement of other organs. It has been shown that mumps virus can infect T cells *in vitro*.

Virus invasion of the CNS presumably occurs across the choroid plexus. It may also occur directly during the phase of haematogenous viraemia. The virus infects the choroidal epithelium as well and multiplies in it. Thus the virus is readily recovered from the CSF during the early phase of meningoencephalitis. The virus may penetrate the brain parenchyma. Once in the neurons, they may spread along the neuronal pathways. Most cases of mumps encephalitis resolve without sequelae. However, rare cases of deafness, obstructive hydrocephalus, and a variety of less common neurological syndromes occur. Mumps meningoencephalitis is an important complication of mumps. At times, it may be the only manifestation of the disease, even in the absence of parotitis. Mumps virus is one of the most common causes of aseptic meningitis. It is benign, and most cases recover without any sequelae.

Mumps virus may persist in the CNS of humans. Mumps virus has been implicated as a causative agent in multiple sclerosis.

Mumps virus causes multi-organ involvement such as orchitis, pancreatitis, oophoritis, mastoiditis, thyroiditis, myocarditis, nephritis and arthritis particularly in post-pubescent children and adults. At least a quarter of all males develop orchitis, unilateral or bilateral, often leading to testicular atrophy and rarely to sterility. From biopsy of the affected testes within the first four days of symptoms, the virus has been isolated. The virus is present in the urine upto two weeks after the onset of the illness. The virus is also excreted in the breast milk.

Though the virus has great potentialities to cause serious manifestations, during childhood mumps is generally a self-limiting disease. The incidence of residua is quite low. Approximately 30 per cent of all mumps infections are subclinical. Mumps virus infection is rare in children under the age of nine months.

Mumps in the first trimester of pregnancy may cause abortion. Transplacental infection has been demonstrated, but teratogenicity has not been observed.

Laboratory diagnosis: The typical case of mumps needs no laboratory confirmation, but it may be essential in atypical infections and where meningitis or orchitis is the sole manifestation. Diag-

nosis may be established by the isolation of the virus and by serological tests. The virus may be isolated from saliva, urine or CSF: from the saliva within 4–5 days, urine upto two weeks and CSF 8–9 days, after the onset of the illness. The urine has to be cleared of debris and then ultracentrifuged. The prepared specimen is inoculated into the amniotic cavity of 6–8 day old chick embryos or primary human or monkey kidney cell cultures. After incubation for 3–5 days, the growth of the virus can be demonstrated in the amniotic fluid by haemagglutination and in tissue cultures by haemadsorption. Immunofluorescence techniques provide direct confirmation of the presence of an isolate in the culture and, if appropriate reagents are used, specify the isolate as mumps.

Serological diagnosis consists of the examination of paired serum samples by complement fixation, haemagglutination inhibition and neutralisation tests, ELISA, RIA and Radial haemolysis test and demonstration of a rise in titre of antibodies to the virus. Complement fixing antibodies to the S antigen appear within 2–3 days, reach a peak in ten days and disappear in 8–9 months. Antibodies to V antigens appear only about the tenth day and they persist for years. Therefore, the former help in detecting recent infection. Haemolysis in-gel assays, and, demonstration of virus specific IgM and IgG levels on a single phase acute serum, when paired sera are unavailable, as a reasonably sensitive alternative can also be done. Delayed hypersensitivity to the virus can be shown by a skin test, but as it becomes positive only sometime after the acute stage of the disease and as hypersensitivity persists for several years, it is of no use as a diagnostic test. Skin testing, however, can be used to detect susceptible persons.

Epidemiology: Man is the only natural host for mumps virus, though experimental disease can be induced in monkeys. The disease is endemic throughout the world and has the highest incidence in children 5–15 years old. Outbreaks may occur in school and army camps.

The virus is shed in the saliva and urine, though the epidemiological significance of the latter is not clear. A patient is infectious from about seven days before till about nine days after the onset of symptoms. Close contact appears to be necessary for the transmission of infection. Inapparent infections are frequent. One infection confers solid immunity as only one antigenic type of the virus exists.

Prophylaxis: As mumps is a mild, self-limited disease, routine prophylaxis may not be very important. For passive prophylaxis in contacts, normal human gamma globulin is not useful, but gamma globulin prepared from the mumps convalescent serum may be effective.

For active immunisation, a killed vaccine prepared from the virus grown in the allantoic cavity of chick embryos was used formerly. This has been replaced by the more effective live attenuated (Jeryl-Lynn strain) vaccine grown in chick fibroblast culture. This is administered either alone, or in combination with live measles and rubella vaccines. A live vaccine has been developed which can be sprayed into the mouth, inducing substantial protection, without any side effects. Live vaccine should not be given to pregnant women, though there is no evidence that mumps virus can damage the fetus.

Newcastle disease virus

This is a natural pathogen of poultry in which it causes explosive outbreaks with high mortality. In India, it is known as the Ranikhet disease virus. Control measures consist of vaccination and slaughter of infected birds. The virus can cause a mild, self-limited conjunctivitis in poultry workers and other persons in contact with the infected birds.

Parainfluenza viruses

The parainfluenza viruses are a group of antigenically related paramyxoviruses which cause respiratory infections in children, and less often in

adults. They also cause natural infection in several species of animals. The parainfluenza viruses are spherical, 125–250 nm diameter, enveloped RNA viruses. The outer envelope is composed of proteins and lipids. Two glycoproteins protrude from the envelope as 10 nm spikes. One of these spikes has haemagglutinin and neuraminidase activities, the HN protein, the other protein mediates fusion and haemolytic properties of the virus and is known as F. protein. The RNA is single stranded. Growth in eggs is poor or absent, except in the case of the Sendai virus. They grow well in primary human or monkey kidney cell cultures. Cytopathic effects are not prominent and their growth in cell cultures is recognised by haemadsorption. The virus is isolatable from nasal washings. They are labile viruses, easily inactivated by heat and by ether. They are antigenically stable. Serological erossreactions exist between the different parainfluenza viruses and with mumps virus. They have been classified into four serotypes (Table 56.1).

The first parainfluenza virus to be identified was the 'Sendai virus' formerly also called the 'haemagglutinating virus of Japan'. It was isolated in 1952 by intranasal inoculation in mice of the lung tissues of children dying of pneumonia in Sendai, Japan. It grows well in the allantoie cavity of eggs and was for a time thought to represent a new type of influenza virus (influenza virus type D). The Sendai virus was subsequently shown to be a widespread natural parasite of mice. Isolation from human cases by mouse inoculation may, therefore, have been only an accidental 'pick up' of the murine virus. Antibodies to the Sendai virus were found prevalent in human sera throughout the world. This observation was explained when an antigenically identical virus was isolated, in 1958, from children with respiratory infection, by the technique of haemadsorption in tissue culture. This virus was called the 'haemadsorption virus type 2' (HA-2). Both these viruses are now classified as the parainfluenza virus type 1, the Sendai virus representing the murine and HA-2 the human varieties. Unlike the Sendai virus, HA-2 does not grow in eggs and has to be isolated in cell cultures.

Parainfluenza virus type 2 was originally isolated in 1955 from children with acute laryngotracheobronchitis or 'croup'. It grows in monkey kidney cell cultures, producing a syncytial cytopathic effect. It was originally termed the 'croup associated' (CA) virus. antigenically similar viruses (simian viruses 5 and 41) cause natural infection in monkeys.

Parainfluenza virus type 3 was originally isolated in 1958 in tissue culture from children with respiratory disease and was called the 'haemadsorption virus type 1' (HA-1). An antigenically identical virus (SF-4) causes a respiratory infection in cattle known as 'shipping fever'.

Parainfluenza virus type 4 was isolated in 1960 from cases of mild respiratory infection. Two antigenic subtypes — A and B — of this virus have been recognised.

Parainfluenza viruses are probably responsible for about 10 per cent of respiratory infections in children who need hospitalisation. The most serious clinical disease is croup which is caused most frequently by types 1 and 2. Type 3 causes lower respiratory disease, including bronchitis, bron-

TABLE 56.1

Serotypes of parainfluenza viruses

Parainfluenza virus type 1	
a. Murine	(Sendai virus)
b. Human	(Haemadsorption virus type 2)
Parainfluenza virus type 2	(Croup-associated virus)
Parainfluenza virus type 3	(Haemadsorption virus type 1)
Parainfluenza virus type 4	(Subtypes A and B)

chiolitis and pneumonia. Type 4 causes minor respiratory illness. In adults, parainfluenza viruses cause milder respiratory infections in which sore throat and hoarseness of voice are common. Rarely, they cause parotitis.

Epidemiology: These are ubiquitous viruses. Parainfluenza type 3 infection is often experienced in the first year of life with 50 per cent of the children being seropositive by 12 months of age while parainfluenza virus type 1 and 2 cause disease in the pre-school child. Type 3 is more endemic in the community, than types 1 and 2 which occur as epidemics. Reinfection occurs not infrequently with all parainfluenza viruses, although the infection is less severe. There is a great tendency for nosocomial spread in paediatric patients. There is little evidence of viraemia or spread by extra-respiratory routes. Virus shedding by patients occurs for 8–11 days.

Respiratory syncytial virus

New born & infants -

The first strain of this virus was isolated in 1956 from chimpanzees with coryza and was called 'the chimpanzee coryza agent' (CCA). A year later, the virus was isolated from children with lower respiratory tract infection. Because it caused cell fusion and the formation of multinucleated syncytia in cell cultures, it was termed the 'respiratory syncytial virus' (RSV). It is now recognised to be the most important cause of lower respiratory tract infection in infants particularly in the first few months of life.

RSV is pleomorphic and ranges in size from 150–300 nm in diameter. Virions appear as membrane-bounded particles with short, closely spread projections. The bilayer membrane encloses an unsegmented RNA. The envelope of RS virus contains two surface glycoproteins. Glycoprotein G mediates attachment to target cells and glycoprotein F is the viral fusion glycoprotein. The F protein directs fusion of viral and cellular membranes, resulting in viral penetration. It can direct fusion of infected cells with adjoining cells resulting in the formation of syncytia. Syncytia formation in addition to being a prominent cytopathic effect, is an additional mechanism of viral spread. The virus possesses neither a haemagglutinin nor a neuraminidase. It is a highly labile virus but can be stabilised by $MgSO_4$. It does not grow in eggs or in laboratory animals. It grows in most types of cultured human cells (including HeLa, HEp-2) and in monkey kidney cells. It is antigenically stable, but minor antigenic variants occur, and at least four 'subtypes' have been identified.

RSV is responsible for nearly half the cases of bronchiolitis and a quarter of all pneumonia occurring during the first few months of life. Occasionally, the virus infection may cause sudden death in infants. Children with a background of allergy are prone to serious disease. The maternal antibodies to the virus are believed to be responsible for inducing an Arthus type of response in infected infants. In adults, infection may be asymptomatic or result in afebrile rhinitis or 'common cold'. In the aged, infection may cause exacerbation of bronchitis.

The incubation period is 4–5 days. At the beginning of the illness the virus multiplies in the nasopharynx. The spread of the virus from the upper to the lower respiratory tract is believed to be via the respiratory epithelium or through aspirated secretions. The virus is capable of cell to cell spread without emergence into extracellular fluid. Viraemia has not been detected. Lack of CMI leads to persistent infection.

Nosocomial infections occur in nurseries and paediatric hospital wards. Transmission occurs primarily via the hands of staff members. Washing of hands after every patient contact, wearing gowns and gloves, and isolation of infected patients reduce nosocomial spread.

Laboratory diagnosis may be made by isolation of the virus, demonstration of the viral antigen or by serology. Virus shedding from the respiratory tract occurs for 1–3 days before the onset of illness and continues for 4–7 days after. Isolation is difficult as the virus is extremely labile and so specimens have to be inoculated directly into tissue cultures without preliminary freezing and thawing.

HeLa, HEp-2 and KB cells are usually employed. Growth is slow and it may take upto two weeks for the typical cytopathie effects to be evident.

A rapid diagnosis can he made by demonstrating the virus antigen by immunofluorescence in the exfoliated cells from the nasopharynx and in cell cultures inoculated with infected secretions. ELISA has proved very reliable and useful in detecting the virus. Other techniques such as electron microscopy to detect viral particles, RIA to detect viral antigen have been developed. RPH has proved the most sensitive compared to IF and virus isolation. Rise in titre of antihody may be demonstrated by complement fixation, neutralisation and ELISA tests, and have proved useful in the retrospective diagnosis of RSV infections.

A formolised vaccine was tried, but vaccinated infants developed a more serious infection than the unvaccinated, when exposed to infection subsequently. This paradoxical response is probably due to the Arthus type reaction induced by the circulating antibodies. No vaccine is available at present.

Ribavarin administered as a continuous aerosal for 3–6 days was clinically beneficial to hospitalised infants. Viral shedding was also decreased.

Measles *(Rubeola)*

Measles probably appeared first after 2500 BC in the *Middle Eastern river valley* civilisations. The first written account of the disease is attributed to Rhazes, 10th century AD, though he quoted outbreaks of the 7th century AD. Throughout the Middle Ages, measles and smallpox were confused as a single disease. It was only in 1629 that measles was considered as a separate entity. Thomas Sydenham in 1690 gave the first clear and accurate description of measles in the English language. In 1846, an epidemic appeared in *Faroe Islands* and it was studied in detail and well described by a Danish medical student Peter Panum.

Measles is the commonest and the most infectious of childhood exanthemata. The viral etiology of measles was established in 1911 hy transmitting the disease to monkeys by inoculation of filtrates of blood and nasopharyngeal secretions from patients. The virus was isolated in monkey and human kidney cell cultures by Enders and Peebles in 1954.

Properties of the virus: The virus resembles paramyxoviruses morphologically. The virus consists of pleomorphic particles 120–250 nm in diameter. The envelope is covered with projecting peplomers of haemagglutinin. The viral envelope is a lipoprotein membrane 10–20 nm in thickness that contains proteins associated with haemagglutinating, haemolytic, and membrane fusion properties of measles viruses. A tightly coiled helical nucleocapsid composed of protein and the single stranded RNA genome are enclosed within the envelope (Fig. 56.1). It possesses a haemagglutinin active against monkey erythrocytes. The virus does not elute from the cells as it is devoid of neuraminidase. It is haemolytic. The virus does not grow in eggs, but it can be grown in human embryonic kidney or amnion cell cultures. Cytopathic effect consists of multinucleate syncytium formation, with numerous acidophilic nuclear and cytoplasmic inclusions. Multinucleate giant cells (Warthin-Finkeldy cells) are also found in lymphoid tissues of patients.

The virus is readily inactivated by heat, ultraviolet light, ether and formaldehyde. It can be stabilised by molar $MgSO_4$ so that it resists heating at 50°C for one hour.

The measles virus is antigenically uniform. It shares antigens with the viruses of canine distemper and bovine rinderpest.

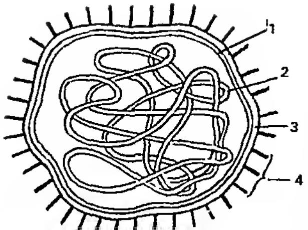

Fig 56 1 Measles Virus 1. Membrane (M) protein 2 Nucleocapsid 3. Lipoprotein membrane 4. Spikes-Haemagglutinin.

tients recover uneventfully, but some develop complications which may be due to the virus (croup or bronchitis) or to secondary bacterial infection (otitis media or pneumonia). Rarely, a fatal giant cell pneumonia may develop, particularly in children with immunodeficiencies. The most serious complication is meningoencephalitis, which has an incidence of about 15 per cent. Many survivors have neurological sequelae. Protracted diarrhoea is often seen as a complication of measles in children in the poor nations. The virus may be recovered from the stools of patients with measles enteritis. A rare late complication is 'subacute sclerosing panencephalitis' (SSPE) A particularly severe form of measles is seen in Africa and some other tropical areas. This may be related to the malnutrition so common in children in these areas. There occurs a suppression of delayed hypersensitivity after measles infection, which may last for several weeks to a few months. Mantoux and other allergic skin tests may be negative during this period. Underlying tuberculosis may become worse following an attack of measles. Recovery from measles may also be associated with an improvement of allergic eczema or asthma and induction of remission in leukaemia, Hodgkin's disease or lipoid nephrosis. Measles induces labour in 22–37 per cent of pregnant women, resulting in spontaneous abortion or premature delivery. There is evidence that the virus crosses the placenta and infects the foetus during maternal measles.

Atypical measles: The introduction of killed measles vaccine in 1965 gave rise to a new clinical syndrome in children who had a history of receiving the vaccine. The syndrome called atypical measles was associated with measles virus infection and was characterised by high fever, pneumonia and an unusual rash (raised papules, wheals and tiny haemorrhages in the skin) without Koplik's spots. Since the stoppage of the use of the killed vaccines, this syndrome has disappeared, except in those who had received killed vaccine as children.

Laboratory diagnosis: In a typical case of measles, the diagnosis is self-evident. But in atypical cases, and for differentiation from rubella, laboratory tests are useful.

A simple diagnostic test, which can be used even before the rash appears, is the demonstration of multinucleated giant cells in Giemsa stained smears of nasal secretions. Measles virus antigen can be detected in these cells by immunofluorescence.

The virus can be isolated, with some difficulty, from the nose, throat, conjunctiva and blood during the prodromal phase and upto about two days after appearance of the rash Virus may be obtained from the urine for a few more days. Primary human embryonic kidney and amnion cells are most useful. Cytopathic changes may take upto a week to develop, but earlier diagnosis of virus growth is possible by immunofluorescence.

Serological diagnosis: Specific neutralisation, haemagglutiniation inhibition and complement fixing antibodies develop early, with maximal titres about the time of onset of rash. A fourfold rise in titre is looked for. Demonstration of measles specific IgM in a single specimen of serum drawn beetween one and two weeks after the onset of the rash is confirmatory. False negatives may occur if the serum is taken earlier than one week or later than two weeks of the onset of the rash.

Epidemiology: Measles is endemic throughout the world and produces epidemics every 2–3 years. Epidemics are usually seen in late winter and early spring, with a peak in April. The disease has maximum incidence in children 1–5 years of age. It is uncommon in the first six months of life due to the presence of maternal antibody. One attack confers solid immunity.

Man is the only natural host for measles. Monkeys are often found infected but they seem to acquire the infection from man. Patients are infectious from three days prior to the onset of symptoms until the rash desquamates. Infectivity is maximum at the prodrome and diminishes rapidly with the onset of rash. Spread is by direct contact with respiratory secretions and aerosols created by coughing and sneezing. The virus enters the body through the respiratory tract and conjunctiva. In the nonimmune, infection almost always results in clinical disease.

In remote islands, the population may be highly susceptible to measles. When the virus is introduced into such communities, it may induce epidemics with high mortality. A classical example was observed in the Faroe Islands where measles appeared in 1846 after an absence of some 60 years. The epidemic spared only the very old persons who had been alive during the previous epidemic. When Greenland had its first exposure to measles virus in 1951, the epidemic affected 99.9 per cent of the indigenous population.

Prophylaxis: Normal human gamma globulin given within six days of exposure can prevent or modify the disease, depending on the dose. This is valuable in children with immunodeficiency, pregnant women and others at special risk.

A live attenuated vaccine was developed by passaging the 'Edmonston' strain of measles virus successively through human kidney and amnion cell cultures, chick embryo amnion and, finally, chick embryo tissue culture. This attenuation was insufficient and the 'Edmonston' vaccine gave rise to a mild attack of measles in children. This 'vaccination measles' could be prevented by the simultaneous administration of a small dose of gamma globulin at a different site. Further attenuation of the 'Edmonston' strain by Schwartz yielded a more innocuous vaccine which produced mild reactions in a minority of recipients only. The further attenuated vaccine, now being used, is virtually free from side effects.

A disadvantage of this vaccine is that it does not induce adequate antibody response in young babies who possess maternal antibodies to measles. Therefore, in advanced countries, the vaccine is administered only at or after 15 months of age. But in India and other developing countries, measles constitutes a major problem even during the first year of life. Therefore, here the vaccine is recommended as early as at nine months of age. One subcutaneous injection is adequate and no booster is needed. It may be given simultaneously with live polio vaccine or other vaccines, if desired, without impairing antibody response to any of them. But if given separately, there should be an interval of at least one month between the administration of the measles vaccine and any other live virus vaccine. A combined measles mumps and rubella (MMR) vaccine is now in wide use. Adverse reactions include mild fever and minimal rash. Though a few cases of SSPE have occurred in children who had received measles vaccine, it has been stated that the association of SSPE with the vaccine (about one case per million doses) is far less than the association with natural measles (5–10 cases per million patients). Contraindications include any febrile illness, untreated active tuberculosis, systemic malignancies, immunodeficiencies and immunosuppression. It is not recommended during pregnancy. The incubation period for the vaccine virus is about 3–5 days, much less than the incubation period of natural measles. Therefore the vaccination of children exposed to infection within upto five days previously can protect them from developing the disease.

The measles vaccine has been extensively used in the USA where it has led to a virtual eradication of indigenous measles.

Sabin and his colleagues have developed another live attenuated vaccine which can be given by intranasal aerosol in young babies and

induces good antibody response irrespective of the presence of maternal antibodies. This may perhaps be the answer for prophylaxis of measles in very young infants.

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57 Arboviruses

Arboviruses (*A*rthropod-*bo*rne viruses) are viruses of vertebrates biologically transmitted by haematophagous insect vectors. They multiply in blood sucking insects and are transmitted by bite to vertebrate hosts. Insect viruses, and viruses of vertebrates that are sometimes mechanically transmitted by insects do not come into this category. Inclusion in this group is based on ecological and epidemiological considerations and hence it contains members that are in some cases dissimilar in other properties. With better understanding of the physical and chemical properties of individual viruses, it has been suggested that they be reassigned to better defined taxonomic groups. Though taxonomically unacceptable, the name 'arbovirus' is a useful biological concept.

Taxonomically, Arboviruses belong to families as diverse as Togaviridae, Bunyaviridae, Reoviridae Arenaviridae and Rhabdoviridae. Most arbovirses of medical importance are togaviruses; some are bunyaviruses and a few are orbiviruses or rhabdoviruses (Table 57.1).

Togaviruses (from *toga*, meaning mantle) are spherical viruses, 40–70 nm in diameter, with lipoprotein envelope and single stranded RNA genome. The togavirus family contains the following genera of medically important viruses:

Alphavirus: (Arbovirus Group A; the name Alphavirus being derived from 'alpha' for the letter A).

Flavivirus: (Arbovirus Group B; the name being derived from *flavi*, meaning yellow, to refer to yellow fever, the most important virus in this group).

Rubivirus (Rubella virus): While the rubella virus possesses morphological and physicochemical features typical of togaviruses, it is antigenically and epidemiologically unrelated to arboviruses. Hence it is not considered in this chapter.

The next important group of arboviruses belong to the family Bunyaviridae.

Bunyaviruses are larger (90–100 nm), fragile, enveloped, spherical viruses, resembling orthomyxovirus in structure. The name is derived from Bunyamwera, a place in Uganda from where the type species, the Bunyamwera virus, was isolated.

They are very labile, being readily inactivated at room temperature. Infectivity may be retained hy storage at −70°C or by lyophilisation. They are susceptible to bile salts, ether and other lipid solvents.

Arboviruses have a very wide host range including many species of animals and birds. The ability to multiply in arthropods is their special characteristic. In the laboratory, mice are commonly employed for growing arboviruses, intracerebral inoculation in suckling mice being the most sensitive method for their isolation. They can be grown in yolk sac or on the chorioallantoic membrane of chick embryos, in tissue cultures of primary cells like chick embryo fibroblasts or continuous cell lines like vero or HeLa, and in cultures of appropriate insect tissues.

Most arboviruses agglutinate red cells of goose or day old chicks. Haemagglutination is influenced by pH and temperature, the optimal requirements varying with different viruses. Spon-

TABLE 57.1

A list of Important Arboviruses

I. GROUP A (Alphavirus)
(All Mosquito borne)
1. *Encephalitis viruses*
Eastern Equine Encephalitis
Western Equine Encephalitis
Venezuelan Equine Encephalitis
2. *Causing fever*
Chikungunya
O' nyong-nyong
Mayaro
Semliki Forest
Sindbis
Ross River

II. GROUP B (Flavivirus)
A. Mosquito borne:
1. *Encephalitis viruses*
St Louis Encephalitis
Ilheus
West Nile
Murray Valley Encephalitis
Japanese Encephalitis
2. Yellow Fever
3. Dengue, types 1,2,3,4
B Tick borne:
1. *Encephalitis viruses*
Russian Spring Summer Encephalitis complex
Louping Ill
Powassan
2. *Haemorrhagic Fever*
Kyasanur Forest Disease
Omsk Haemorrhagic Fever

III. BUNYAVIRIDAE
1 *Bunyamwera group*
Bunyamwera
Batai
Hesha
Germiston
2. *Bwamba Group*
Bwamba
Pongola
3. *C Group* (Formerly Arbovirus Group C)
Apeu
Caraparu
4. *California group*
California encephalitis
La Crosse
Tahyna

5. *Simbu Group*
   Sathuperi
   Kaikalur
   Oropouche
   Ingwavuma
6 *Turlock Group*
   Turlock
   Umbre
7 *Crimean-Congo Haemorrhagic Fever Group*
   Crimean-Congo Haemorrhagic Fever
   Hazara
8. *Phlebotomus Fever Group*
   Sandfly Fever
   Karimabad
9. *Nairobi Sheep Disease Group*
   Nairobi Sheep Disease
   Ganjam
10 *Ungrouped viruses*
   Rift Valley Fever
   (Many more groups have been described)

IV. ORBIVIRUS (REOVIRIDAE)
~~African Horse Sickness~~
Colorado Tick Fever
Palyam
Kasba
Vellore

V. VESICULOVIRUS (RHABDOVIRIDAE)
Chandipura

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taneous elution does not occur. Haemagglutination is inhibited specifically by antibody and nonspecifically by lipoprotein inhibitors in serum, brain and other tissues.

Antigenic structure and classification: Three antigens can be identified — haemagglutinins, complement fixing and neutralising antigens, all integral parts of the virus particle. Considerable antigenic cross reactions occur among arboviruses. Based on antigenic relationships in haemagglutination inhibition and complement fixation tests, the 474 odd arboviruses identified so far have been classified into some 54 antigenic groups and 90 odd ungrouped viruses. About 90 arboviruses are associated with disease in man. In India, 41 arboviruses have been detected of which some ten are known to cause human disease.

Pathogenesis: The virus enters the body through the bite of the insect vector. After multiplication in the reticuloendothelial system, viraemia of varying duration ensues and in some cases, the virus is transported to the target organs, such as the central nervous system in encephalitides, liver in yellow fever and capillary endothelium in haemorrhagic fevers.

Arboviruses cause the following clinical syndromes; fever with or without rash and arthralgia; encephalitis; haemorrhagic fever; and the characteristic systemic disease, yellow fever (Table 57.2). All infections occur in varying degrees of severity, subclinical infections being very common.

Arboviruses also cause a number of veterinary diseases such as Eastern, Western and Venezuelan equine encephalitis in horses in America, Rift Valley fever in sheep and cattle in Africa,

TABLE 57.2

Arboviruses associated with different clinical syndromes

Virus	Genus	Distribution	Vector	Reservoir
FEVER, WITH OR WITHOUT RASH AND ARTHRALGIA				
Chikungunya	Alphavirus	Africa, Asia	Mosquito	Not known; ? Monkeys.
O'nyong-nyong	Alphavirus	Africa	Mosquito	Not known
Ross River	Alphavirus	Australia	Mosquito	Small mammal.
Sindbis	Alphavirus	Africa, Asia, Australia	Mosquito	Birds, Mammals.
Mayaro	Alphavirus	South America	Mosquito	Monkeys, Marsupials.
Dengue, types 1-4	Flavivirus	Widespread, especially Asia, Pacific, Caribbean	Mosquito	Not known, ? Monkeys
West Nile	Flavivirus	Africa, Asia	Mosquito	Birds
Sandfly fever	Bunyavirus	Mediterranean, Asia, Tropical America	Sandfly	Not known, ? Small mammals
Rift-Valley Fever	Bunyavirus	Africa	Mosquito	Sheep, Cattle.
Oropouche	Bunyavirus	South America	Mosquito	Not known
Colorado tick fever	Orbivirus	USA	Tick	Rodents.
ENCEPHALITIS				
Eastern Equine encephalitis	Alphavirus	Americas	Mosquito	Birds
Western Equine encephalitis	Alphavirus	Americas	Mosquito	Birds, ? Reptiles.
Venezuelan Equine encephalitis	Alphavirus	Americas	Mosquito	Rodents
St Louis encephalitis	Flavivirus	Americas	Mosquito	Birds
West Nile	Flavivirus	Africa, Europe, West Asia	Mosquito	Birds
Japanese encephalitis	Flavivirus	East Asia	Mosquito	Birds
Murray Valley encephalitis	Flavivirus	Australia	Mosquito	Birds
Russian Spring Summer encephalitis complex	Flavivirus	East Europe, USSR	Tick	Rodents, other mammals, Birds, Ticks.
Louping Ill	Flavivirus	Britain	Tick	Sheep
Powassan	Falvivirus	North America	Tick	Rodents
California encephalitis	Bunyavirus	North America	Mosquito	Rodents
HAEMORRHAGIC FEVER				
Chikungunya	Alphavirus	Africa, Asia	Mosquito	Not known, ? Monkeys
Dengue, types 1-4	Flavivirus	Tropics	Mosquito	Not known, ? Monkeys.

Yellow fever	Flavivirus	Africa, South America	Mosquito	Monkeys, Man.
Kyasanur Forest disease	Flavivirus	India (Karnataka)	Tick	Rodents, ? Ticks.
Omsk haemorrhagic fever	Flavivirus	USSR	Tick	Small mammals.
Crimean-Congo haemorrhagic fever	Bunyavirus	USSR, Central Asia, Africa	Tick	Small mammals.

blue tongue in asses in India, Africa and America, Ganjam disease of sheep in India and African horse sickness in horses and mules in Africa and Asia.

Laboratory diagnosis: Diagnosis may be established by virus isolation or serology. As all arbovirus infections are viraemic, blood collected during the acute phase of the disease may yield the virus. Isolation may also be made from CSF in some encephalitic cases, but the best specimen for virus isolation is the brain. Specimens are inoculated intracerebrally into suckling mice. The animals develop fatal encephalitis, though serial blind passages may be necessary in some cases. Some viruses may also be isolated in tissue cultures or, less readily, in eggs. Isolates are identified by haemagglutination inhibition, complement fixation, gel precipitation, immunofluorescence or neutralisation with appropriate antisera. Virus isolation from insect vectors and reservoir animal or avian species also aids identification of arbovirus activity in the area.

Diagnosis may also be made serologically by demonstrating rise in antibody titre in paired serum samples by haemagglutination inhibition, complement fixation or neutralisation tests. Serological diagnosis is often complicated due to the antigenic cross reaction between related viruses. This is especially so in the sera of persons who had prior infection or immunisation with other arboviruses because of the broad antigenic reactivity in such cases.

Epidemiology: The epidemiology of arbovirus infections is linked with the ecology of their arthropod vectors and vertebrate hosts. Most arboviruses exist in nature in animal or avian species in which infection is asymptomatic. Infection is maintained in these species by a silent cycle involving mosquitoes or other arthropods naturally feeding on these species. The vector arthropod gets infected by biting a viraemic vertebrate. The vector, in turn, becomes infective only after an incubation period during which the virus multiplies in its body to a sufficiently high titre (extrinsic incubation period). Human disease results only when the virus is accidentally transfered to man either directly by the vector or through the intermediary of animal reservoirs. A second epidemiological pattern is seen in diseases like dengue where no nonhuman vertebrate host has been identified. Here, the virus is maintained in a cycle composed of man and the domestic mosquito. In certain tick borne infections, the virus may be maintained for considerable periods by transovarial transmission in ticks before it finds a vertebrate host.

Control: Control measures are indicated only in those infections that lead to epidemics or epizootics. These consist essentially of vector control and immunisation. While vaccination is very effective in yellow fever, it has not been of equal value in the control of other arbovirus diseases. Administration of hyperimmune serum has been shown to be effective in some cases experimentally, but this has little clinical application.

Group A arboviruses *(Alphavirus)*

Group A arboviruses currently comprise 25 serotypes, all of them mosquito borne. They exhibit antigenic cross reaction in haemagglutina-

tion inhibition and to a lesser extent in complement fixation tests. The neutralisation test is more specific. They produce epidemics of encephalitis in America and dengue-like fever in the tropics.

Encephalitis viruses: Three members of this group, Eastern, Western and Venezuelan equine encephalitis viruses, cause encephalitis in horses and man. Eastern equine encephalitis (EEE) occurs along the eastern sea board of the USA and causes sporadic cases and small epidemics. Western equine encephalitis (WEE) is more widely distributed in America and causes large epidemics. Venezuelan equine encephalitis (VEE) prevalent in Central and South America usually causes an influenza-like illness, with encephalitis in a small proportion of cases. Several species of *Culex* and *Anopheles* mosquitoes are the vectors, and wild birds the reservoirs. Formalinised vaccines have been developed for EEE and WEE and a live attenuated vaccine for VEE.

Viruses causing febrile illness: 1. Chikungunya virus: This virus was first isolated from human patients and *Aedes aegypti* mosquitoes from Tanzania in 1952. The name 'chikungunya' is derived from the native word for the disease in which the patient lies 'doubled up' due to severe joint pains. Epidemics of chikungunya have occurred in many African countries In 1958, the virus caused a large epidemic of haemorrhagic fever in Thailand. The virus first appeared in India in 1963, when along with dengue, it caused very extensive epidemics in Calcutta, Madras and other areas subsequently. Chikungunya outbreaks have occurred at irregular intervals along the east coast of India and in Maharashtra till 1973. Since then the virus has been quiescent.

The disease presents as a sudden onset of fever, crippling joint pains, lymphadenopathy and conjunctivitis. A maculopapular rash is common and some show haemorrhagic manifestations. Haemorrhagic lesions were common in Calcutta when the disease first appeared there in 1963, but have been extremely rare afterwards. The fever is typically biphasic with a period of remission after 1–6 days of fever. The vector is *Aedes aegypti*. No animal reservoir has been identified. Antibody to the virus has been demonstrated in horses, cattle and other domestic animals, but its significance is not known. No vaccine is available.

2. O'nyong-nyong virus: This virus was first isolated in Uganda. This is confined to Africa, is closely related to the chikungunya virus antigenically and causes a similar disease. This is transmitted by the *Anopheles* species. The Mayaro virus causes a similar disease in the West Indies and South America.

3. Semliki Forest virus, first isolated in 1942 in Uganda from Aedes mosquitoes has not been associated with clinical illness in man though neutralising antibodies to the virus have been demonstrated in Africans. The Sindbis virus, originally isolated from *Culex* mosquitoes in the Sindbis district of Egypt in 1952, has subsequently been recovered from other parts of Africa, India, Philippines and Australia. In Africa, it is known to be associated with febrile illness in man. In India, antibodies have been detected in human sera, but no association has been established with human disease. The closely related Ross River virus has been associated with epidemic polyarthritis in Australia.

Group B arboviruses *(Flavivirus)*

This is the largest and the most important group of arboviruses containing over 60 serotypes. Representative members of this group are distributed in all parts of the world, covering all the zoogeographic regions. They may be considered under two sections, the mosquito borne and the tick borne viruses.

MOSQUITO BORNE GROUP

1. Encephalitis viruses: Five members of this group cause encephalitis, each of them limited to a geographic zone.

a. St. Louis encephalitis virus: This is prevalent in North and Central America and is the most important mosquito borne disease in the USA. It

has caused several large epidemics in recent years, the clinical picture ranging from mild febrile illness to frank encephalitis and the case fatality ranging from two to twenty per cent. Wild birds act as the reservoir and *Culex tarsalis* as the vector.

b. Ilheus virus: This occurs in South and Central America, maintained in forests by a cycle involving mosquitoes, wild birds and monkeys. Human infection is largely subclinical or leads to febrile illness. Encephalitis is rare.

c. West Nile virus: This virus, originally isolated in 1937 from the West Nile province of Uganda, has since been reported from many African countries, Israel, Cyprus, France and India. It causes a dengue-like illness in man and encephalitis in horses. It is endemic in Egypt, affecting mainly children. In Israel it causes epidemics. The virus is maintained in nature in wild birds by *Culex* mosquitoes.

In India, the virus has been isolated from *Culex* mosquitoes and from a patient with fever. Its importance as a pathogen was confirmed following its isolation from the brains of three fatal cases of encephalitis in children in Karnataka during 1980–81. West Nile virus may perhaps be contributing to sporadic and epidemic encephalitis in India, assumed to be caused by Japanese encephalitis virus.

d. Murray Valley encephalitis virus: This is confined to Australia and New Guinea. The virus was isolated during an epidemic of encephalitis in the Murray River valley in 1951. Perhaps the same virus had been isolated earlier during the epidemics of encephalitis in 1917–1918, when it went under the name of Australian 'X' disease. The virus is believed to occur normally in an enzootic cycle involving wild birds and mosquitoes, and to break out only occasionally into epidemics. *Culex annulirostris* is the vector.

e. Japanese encephalitis: This virus occurs along the Orient, from Korea and Japan in the north to India and Malaysia in the south. The disease had been recognised in Japan since 1871 and was named Japanese 'B' encephalitis to distinguish it from 'encephalitis A' (encephalitis lethargica, Von Economo's disease) which was then prevalent. The virus was first isolated in Japan during an epidemic in 1935. Several large epidemics have occurred since then. Epidemics show a seasonal incidence (summer-autumn) in the temperate regions, though this is not evident in the tropical areas. *Culex tritaeniorhynchus*, a rural mosquito that breeds in rice fields is the principal vector.

The Japanese encephalitis virus causes the most serious clinical disease among the five viruses of this group. The disease typically has an abrupt onset with fever, headache and vomiting. After 1–6 days, signs of encephalitis set in with nuchal rigidity, convulsions, altered sensorium and coma. The fever is high and continuous. There is neutrophil leucocytosis in the peripheral blood and pleocytosis with normal or raised sugar and a slightly raised protein in the CSF. The mortality rate in some epidemics has been upto 50 per cent. Convalescence may take many weeks. Residual neurological damage may persist in upto 50 per cent of survivors. The large majority of infections are, however, asymptomatic and it has been estimated that 500–1000 inapparent infections occur for every case of clinical disease.

In India, Japanese encephalitis was first recognised in 1955 when the virus was isolated from mosquitoes of the *Culex vishnui* complex from Vellore during an outbreak of encephalitis in Tamil Nadu. The virus continued to be active in Tamil Nadu and Andhra in subsequent years also, causing illness mainly in children, indicating the endemic nature of the virus. Most of the cases occurred between October and November.

Japanese encephalitis remained confined to the southeastern parts of India till 1973, when it caused a large outbreak of encephalitis in West Bengal. The epidemic affected adults also, with mortality rates approaching 50 per cent suggesting that the virus was freshly introduced into the area. Cases occurred mainly during June to October. From 1976, there have been periodical outbreaks of the disease in various parts of India — Dibrugarh (Assam) in the East, Gorakhpur (Uttar Pradesh) in the North and Goa in the

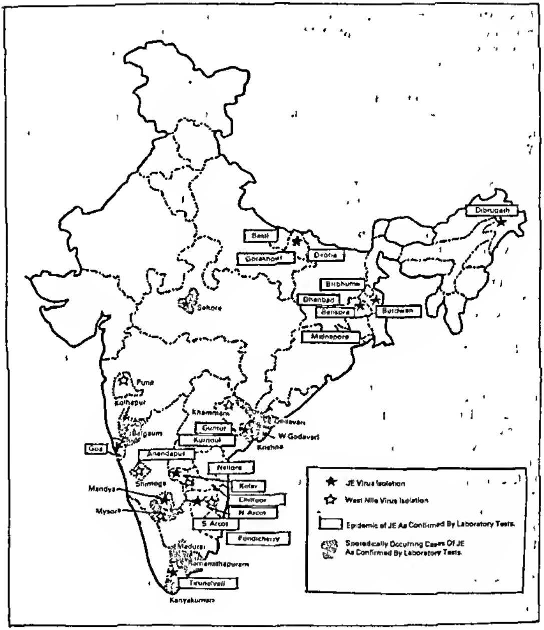

Fig 57.1 Distribution of Japanese encephalitis in India (Courtesy National Institute of Virology, Pune)

West. In the South, outbreaks have occurred in Kolar in Karnataka, various areas in Andhra Pradesh, Tirunelveli and South Arcot in Tamil Nadu, and in Pondicherry. In addition, sporadic cases have been reported from different parts of the country, excepting the northwestern states and Kerala. Japanese encephalitis has become a major public health problem of national importance (Fig 57.1).

The natural cycle of the virus has been worked out in detail in Japan. Herons act as reservoir hosts and pigs as amplifier hosts. Human infection is a tangential 'dead-end' event and occurs when the infected mosquitoes reach high density. The natural cycle in India also may be similar. Natural infection has been demonstrated in Ardeid birds (herons and egrets), as well as bird to bird transmission through *Culex tritaeniorhynchus*. Other birds such as ducks, pigeons and sparrows may also be involved. Vertebrate hosts may include cattle and buffaloes, besides pigs. The major vector *Culex tritaeniorhynchus* has a predilection for the cattle and bites them in preference to man or pigs. The high cattle–pig ratio in the country has been suggested as a factor limiting human infection.

Preventive measures include mosquito control and locating piggeries away from human dwellings. A formalin inactivated mouse brain vaccine using the Nakayama strain has been employed successfully for human immunisation in Japan, and, in a small scale, in India also. Two doses at two weeks' interval, followed by a booster 6–12 months later constitute a full course. Immunity produced by the vaccine is shortlived. Vaccination of pigs has been proposed in view of their importance as amplifier hosts.

2. *Yellow fever*: Yellow fever was recognised as a clinical entity as early as in the 17th century and was familiar to the pirates as 'Yellow Jack'. It is a native of Africa and was transported thence along the trade routes to Europe and America. The most serious epidemics occurred in the Western Hemisphere — Central America and the Caribbean, and even as far north as New York. Since early this century, the disease has been largely confined to certain areas of Africa and South and Central America.

Carlos Finlay in Cuba in 1881 suggested that yellow fever was spread by *Aedes aegypti* mosquitoes. In 1900, the U.S. Army Yellow Fever Commission, under Walter Reed, confirmed this observation and demonstrated that Aedes mosquitoes were infected by feeding on human patients during the early viraemic phase of the disease and became infective after an extrinsic incubation period of 12 days. This led to the prompt eradication of the disease from Cuba and the Panama Canal area by controlling the *Aedes aegypti* mosquitoes. There were even hopes of ultimate total eradication of the disease but these had to be adandoned when in 1932, outbreaks of yellow fever occurred in Brazil in areas devoid of *Aedes aegyptii*. It was then recognised that the virus survives in another cycle — the forest or sylvatic cycle — involving forest animals and mosquitoes.

The yellow fever virus was first isolated in 1927 by inoculating rhesus monkeys with the blood of an African patient named Asibi. The virus was shown by Thelier (1930) to grow well following intracerebral inoculation in mice. The infected mouse brain was used as a vaccine in former French West Africa (Dakar vaccine) though this was encephalitogenic. It was later replaced by a non-neurotropic (17D) vaccine.

After an incubation period of 3–6 days, the disease starts as a fever of acute onset with chills, headache, nausea and vomiting. The pulse is usually slow despite a high temperature. Jaundice, albuminuria, and haemorrhagic manifestations develop and the patient may die of hepatic or renal failure. Most cases are less severe, especially in the endemic areas, and may present as undifferenttiated fever without jaundice.

Histologically, the liver shows cloudy and fatty degeneration and necrosis which is typically midzonal. The necrosed cells coalesce and become hyalinised leading to the formation of charac-

teristic eosinophilic masses known as Councilman bodies. Acidophilic intranuclear inclusion bodies (Torres bodies) may be seen in the infected liver cells in the early stages. The histological picture of the liver is specific enough to be diagnostic, and this was the basis of early surveys undertaken to detect areas of yellow fever activity. A special instrument (viscerotome) was employed for the collection of liver tissues from fatal cases for histological diagnosis.

The epidemiology of yellnw fever was clarifieid only after the recognition that the disease occurs in two distinct patterns. In the urban cycle, man serves both as the natural reservoir and as the definitive case, the virus being transmitted by the domestic *Aedes aegypti* mosquito. In the fotest or sylvatic cycle, wild monkeys act as the reservoirs nnd forest mosquitoes (*Haemogogus spegazzinii* in South America and *Aedes africanus* and *simpsoni* in Africa) ns the vectors. Human cases occur only when man trespasses into the forest or when the monkeys raid villages near the forest.

The control of urban yellow fever can be achieved by eradicating the vector mosquito, as was shown in Cuba and Panama early this century by Gorgas, but this is obviously impracticable with the sylvatic disease. Two very effective vaccines have been developed for human use. The French neurotropic vaccine (Dakar) produced from Infected mouse brain was thermostable and administered by scarification and hence convenient for use under field conditions in the tropical areas. But the vaccine carries a high risk of producing encephalitis in the vaccinees, especially in children. A safe and equally effective vaccine, the 17D vaccine was developed by Theiler in 1937 by passaging the Asibi strain serially in mouse embryo and whole chick embryo tissues and then in chick embryo tissue from which the central nervous tissue has been removed. The 17D vaccine is thermolabile and is administered by subcutaneous inoculation. Vaccination which is mandatory for travel to or from endemic areas is valid for ten years beginning ten days after vaccination. In India, the 17D vaccine is manufactured at the Central Research Institute, Kasauli. Yellow fever is at present confined to Central and South America (between latitudes 15°N and 20°S) and Africa (between 15°N and 15°S). Yellow fever does not exist in India and is important to us for this paradoxical reason. India offers a receptive area with a large population of *Aedes aegypti* and nonimmune humans. Strict vigilance is enforced on vaccination and quarantine for travel from endemic areas. This, no doubt, has checked the entry of the virus into India, but perhaps a more likely reason could be that even the stray virus that may be introduced may not be able to get established in the vectors due to the prevalence in the local *Aedes aegypti* of the Dengue fever virus.

3. Dengue: Dengue virus is widely distributed throughout the tropics and subtropics. Dengue fever which occurs intermittently in large epidemics is clinically similar to fever caused by chikungunya, O'nyong-nyong and West Nile viruses. Four types of the dengue virus exist: Type 1 first isolated from Hawaii in 1944, type 2 from New Guinea in 1944 and types 3 and 4 from the Phillippines in 1956.

Dengue presents typically as a fever of sudden onset with headache, retrobulbar pain, pain in the back and limbs (break-bone fever), lymphadenopathy and maculopapular rash. The fever is typically biphasic (saddle back) and lasts for 5–7 days, followed as a rule by recovery. Dengue may also occur in more serious forms, with haemorrhagic manifestations ('Dengue Haemorrhagic Fever') or with shock ('Dengue Shock Syndrome'). These complications, first recognised in Thailand, have since occurred in many countries in South East Asia and Western Pacific. They are more common in previously healthy children in the indigenous populations of the endemic areas. They are believed to represent a hypersensitivity response to sequential dengue virus infection in persons already sensitised by prior exposure to other serotypes of the virus. Haemorrhagic dengue is seen only rarely in India

The Dengue virus is transmitted from man to

man by the *Aedes aegypti* mosquitoes. No vertebrate host other than man has been identified. There is, however, some evidence that, as in yellow fever, dengue also may have a sylvatic cycle involving monkeys, squirrels and other forest animals.

Dengue is common along the east coast of India and has frequently caused highly widespread epidemics, sometimes along with chikungunya virus. All four types of dengue virus have been isolated in this country. Occasionally, more than one type of dengue virus has been isolated from the same patient.

Control of dengue is limited to vector control as no vaccine is available.

TICK BORNE GROUP

These viruses produce two clinical syndromes, encephalitis and haemorrhagic fevers.

1. Tick borne encephalitis viruses: A number of viruses belonging to the Russian Spring Summer Encephalitis (RSSE) complex cause encephalitis along a wide area of the northern land mass from Scotland to Siberia. The names given to the disease vary from one area to another depending on the variations in the prominent clinical features. Thus, in Scotland, it is called 'louping ill' as the disease occurs primarily in sheep in which it causes a curious 'leaping' gait. Human cases that result from contact with sheep are mild and present as aseptic meningitis. It is called Central European Encephalitis, biphasic meningoencephalitis and RSSE, in Central Europe, Eastern Europe and USSR, respectively. RSSE is the most serious form, with high rates of fatality and permanent paralytic sequelae in some survivors. Infection is transmitted by the bite of Ixodid ticks. The virus is transmitted transovarially in ticks so that they can act as the vector as well as the reservoir hosts. Wild rodents and migrating birds are other reservoirs. Biphasic meningoencephalitis may be transmitted to man by drinking the milk of infected goats. The control of infection by the RSSE complex depends on the avoidance of tick bites. A formalin inactivated RSSE vaccine has been found useful.

Another tick borne virus, the Powassan virus causes encephalitis in Canada and Northern USA.

2. Tick borne haemorrhagic fevers: a. Kyasanur Forest Disease (KFD): This is a haemorrhagic fever that occurs in Karnataka State (India). In 1957, several dead monkeys were noticed in the Kyasanur forest in Shimoga district in Karnataka along with a severe prostrating illness in some of the villagers in the area. A similar illness had been observed in the locality a year earlier also. A new arbovirus, antigenically related to the RSSE complex, was isolated by investigators from the National Institute of Virology (formerly Virus Research Centre), Pune, from the patients and dead monkeys. It was named the KFD virus after the name of the place from where the first isolations were made.

KFD has a sudden onset with fever, headache, conjunctivitis, myalgia and severe prostration. Some cases develop haemorrhages into the skin, mucosa and viscera. The case fatality rate is about five per cent. For many years after its discovery in 1957, the epizootic and epidemic activity of KFD remained confined to the areas contiguous to its original focus in Sagar, Sorab and Shikarpur taluks of Shimoga district. Between 1972 and 1975, a few other smouldering foci developed in the adjacent areas in North Kanara and Shimoga districts. Though the disease had not been observed anywhere else, the virus appeared to be more widely distributed, as antibodies had been demonstrated in man and animals in the Kutch and Saurashtra peninsula and in some other scattered places in India.

The situation changed suddenly in 1982 with the appearance of an epizootic and epidemic in Belthangadi taluk in South Kanara. This followed the clear-felling of part of an evergreen reserve forest in the area in September 1982. The outbreak, known commonly as 'monkey fever', started with dead monkeys being observed in October. The first human case was observed late in

December and during the next five months, 1142 human cases were recorded with 104 deaths. The outbreak subsided with the onset of monsoon in June, but reappeared the following December. The ecological disturbance caused by clear-felling of the virgin forest is believed to have activated a silent enzootic focus of the virus (Fig. 57.2).

Forest birds and small mammals are believed to be the reservoir hosts. Infection is transmitted by the bite of ticks, the principal vector being *Haemaphysalis spinigera.* As infection in monkeys leads to fatal disease, they are unlikely to be the primary reservoirs, but only amplifier hosts. Haemaphysalis ticks may act as the reservoir to some extent as transovarial transmission of the virus has been demonstrated in them.

Though KFD is related antigenically to the RSSE complex, the RSSE vaccine has not been found to confer any protection against the disease. A killed KFD vaccine was used in a small field trial and appeared to provide some degree of protection.

b. Omsk haemorrhagic fever: This occurs in the USSR and Rumania. It is clinically similar to KFD and is caused by a related virus. Dermacentor ticks are the vectors.

Bunyaviridae

This large group of arboviruses contains over 200 species, most of which are mosquito borne. Some are transmitted by sandflies (e.g., Phlebotomus fever group) or ticks (e.g., Crimean Congo Haemorrhagic Fever group). The Hantaan virus causing haemorrhagic fever with renal syndrome and spread through rodent excreta also belongs to this group. Some bunyaviruses are established pathogens, causing natural diseases, and even epidemics and epizootics, while many have been isolated only from insect vectors and have not been associated with any human or animal diseases.

Bunyamwera group: Bunyamwera, Ilesha and Germiston viruses, isolated from Uganda, Nigeria and South Africa, respectively, have been associated with mild fever in patients. The Chittoor virus, isolated from mosquitoes in India has been found to be identical with the Batai virus belonging to this group. Antibodies to the Chittoor virus have been demonstrated in the sera from cattle, sheep, goats, horses and camels in India, but no association has been established with any human or animal disease.

Bwamba group: The Bwamba virus was isolated from febrile patients in Uganda. The closely related Pongola virus was isolated from South Africa.

C group: Several viruses isoalted from a limited area around Belem (Brazil), Trinidad and Panama constitute this group. They cause mild febrile illness.

California group: Members of this group occur in America, Africa and Europe. The California encephalitis virus was isolated from mosquitoes, in 1943. It produces fever with encephalitis or aseptic meningitis. It is a benign illness, fatalities and sequelae being very rare. The closely related La Crosse virus, first isolated from a fatal case of encephalitis in La Crosse, Wisconsin, is widely distributed across the USA. It causes the same clinical picture as California encephalitis. Tahyna virus of this group has been isolated from man in Europe, but its pathogenic role is uncertain.

Simbu group: Oropouche virus belonging to this group causes febrile illness in Trinidad. Sathuperi, Kaikalur and Ingwavuma viruses of this group have been isolated in India from mosquitoes or birds, but are not known to cause human infection.

Turlock group: Umbre virus belonging to this group has been isolated from culex mosquitoes in India.

Crimean-Congo haemorrhagic fever group: The Crimean haemorrhagic fever virus, first isolated

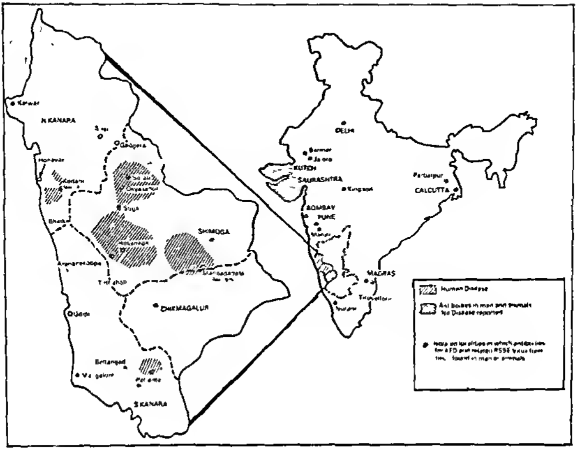

Fig. 57 2 Distribution of Kyasanur Forest Disease (Courtesy: National Institute of Virology, Pune).

in Crimea in 1945, was subsequently found to be identical with the Congo fever virus isolated in 1956 in Zaire (Congo); hence the name Crimean-Congo Haemorrhagic Fever (CCHF). The disease is endemic in Eastern Europe, Central Asia and many parts of Africa. Cattle, Sheep, goats and other domesticated mammals act as natural reservoirs. It is transmitted by *Hyalomma* ticks. During the acute phase of the disease, the blood of patients is highly infectious and direct transmission may occur through contact. A related virus, *Hazara,* has been isolated in Pakistan. It is also widespread in Iran, Iraq and the UAE. Antibodies to CCHF group of viruses have been detected in human and animal sera from India.

Phlebotomus fever group: Phlebotomus fever, also known as sandfly fever, papattaci fever and three-day fever is a self-limited, nonfatal fever transmitted by the bite of the sandfly *Phlebotomus papattaci.* It occurs along the Mediterranean coast and Central Asia, extending as far east as Pakistan and India. Cases have been reported from South and Central America also. Two antigenic types of the virus exist — the Sicilian and Naples strains. Both types have been isolated in India from sandflies and febrile patients. No vertebrate host other than man has been identified. There is some evidence for vertical transmission of the virus in sandflies.

Nairobi sheep disease group: This disease affects

sheep in large parts of equatorial Africa and causes considerable economic loss. The related Ganjam virus causes disease in sheep in India. It appears to be transmitted by *Haemaphysalis* ticks. There is some evidence that it may infect man. Accidental infection in laboratory workers has caused mild febrile illness.

Rift Valley fever: This mosquito borne virus causes enzootic hepatitis in sheep and other domestic animals in Africa and is named after Rift Valley, Kenya, where it was first recognised. It causes periodic epizootics and epidemics. The human disease resembles influenza. In 1978, it caused an extensive epidemic in Egypt with many deaths.

Orbivirus

Colorado tick fever is a self-limited benign illness with a biphasic course, associated with leucopenia. It is spread by the wood tick *Dermacentor andersoni* and the distribution of the disease in Western USA is limited to the habitat of the tick.

African horse sickness virus, transmitted by *Culicoides*, has for long been known to cause disease among equines in Africa. In 1959–60 it spread eastwards in epizootic waves to Iran, Afghanistan, Pakistan and India. It caused extensive disease among army horses and mules in India.

Palyam, Kasba and Vellore viruses, belonging to the orbivirus group, have been isolated from mosquitoes in India, but their pathogenic role is not known.

Vesiculovirus

The Chandipura virus, belonging to the vesiculovirus group of Rhabdoviridae, was isolated in 1967 from the blood of patients during an epidemic of dengue-chickungunya fever in Nagpur. The virus appears to multiply in sandflies and *Aedes* mosquitoes. Antibodies are common in human sera from different parts of India, as well as in animal sera. The pathogenic significance of the virus has not been established.

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58 Rhabdoviruses

Bullet shaped viruses with single stranded RNA genome are classified as rhabdoviruses (from *rhabdos*, meaning rod). The family Rhabdoviridae contains viruses that infect animals, birds, fishes, insects and plants. Some members multiply in vertebrates and arthropods. Rhabdoviruses infecting animals belong to two genera, —*Vesiculovirus* containing vesicular stomatitis virus and related viruses, and *Lyssavirus* containing rabies virus and related viruses. The name lyssavirus is derived from *lyssa*, meaning rage, a synonym for rabies.

RABIES VIRUS

Morphology: Rabies virus is bullet shaped, 180 x 75 nm, with one end round or conical and the other planar or concave. The outer lipoprotein envelope carries spikes. Spikes do not cover the planar end of the virion. Spikes may be released from the envelope by treatment with lipid or detergents. Beneath the envelope is the protein membrane layer which may be invaginated at the planar end. The membrane may project outwards from the planar end of some virions forming a

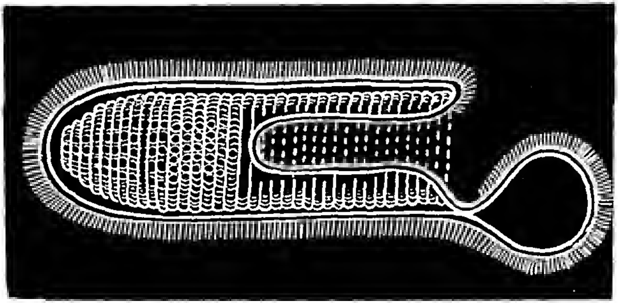

Fig 58.1 Morphology of Rabies virus, shows membrane envelope with surface projections, axial channel, terminal knob and helical ribonucleoprotein capsid.

bleh. The core of the virion consists of helically arranged ribonucleoprotein (Fig. 58.1).

Resistance: The virus is sensitive to ethanol, iodine preparations, quaternary ammonium compounds, and lipid solvents such as ether, chloroform and acetone. It is inactivated by phenol, formalin, beta propiolactone, ultraviolet irradiation and sunlight. Thermal inactivation occurs in one hour at 50°C and five minutes at 60°C. It dies at room temperature, but can survive for weeks when stabilised by 50% glycerol. It survives at 4°C for weeks. It can be preserved at –70°C or by lyophilisation.

Antigenic properties: The surface spikes are composed of glycoprotein, which induces the formation of protective antibodies when injected into animals. The purified glycoprotein may therefore provide a safe and effective subunit vaccine. The spike glycoprotein also induces complement fixing antibodies which may be relevant in the complement dependent immunolysis of cells infected with rabies virus.

Rabies virus possesses haemagglutinating activity, optimally seen with goose erythrocytes at 0°–4° and pH 6.2. Haemagglutination is a property of the virus surface. It is inactivated by heat (56°C for 30–60 minutes.), ether, trypsin, pronase, deoxycholate or Tween 80, but not by beta propiolactone. Haemagglutination inhibiting antibodies develop following infection or immunisation. HI antibodies parallel neutralising antibodies. The HI test would therefore provide a useful method of assaying immunity to rabies, but the low sensitivity of the test and the presence of nonspecific inhibitors in all sera limit its value. Nonspecific inhibitors can be destroyed by treatment with acetone or kaolin.

The nucleocapsid protein induces complement fixing antibodies. These are not protective. The antigen is group specific and cross reactions are seen with some rabies related viruses. Antiserum prepared against the nucleocapsid antigen is useful in diagnostic immunofluorescence tests. Other antigens identified include two membrane proteins, glycolipid and RNA dependent RNA polymerase.

Host range and growth characteristics

1. Animals: All warm blooded animals are susceptible to rabies infection, though differences in susceptibility exist between species. For example, cattle, cats and foxes are highly susceptible, whereas skunks, opossums and fowl are relatively resistant. Man and dogs occupy an intermediate position. Pups are more susceptible than adult dogs. Experimental infection can be produced in any laboratory animal, but mice are the animals of choice. They can be infected by any route. After intracerebral inoculation, they develop encephalitis and die within 5–30 days.

Rabies virus isolated from natural human or animal infection is termed *street virus*. Following inoculation by any route, it can cause fatal encephalitis in laboratory animals after a long and variable incubation period of about 1–12 weeks. Intracytoplasmic inclusion bodies (Negri bodies) can be demonstrated in the brains of animals dying of street virus infection. After several serial intracerebral passages in rabbits, the virus undergoes certain changes and becomes what is called the *fixed virus*. The fixed virus is more neurotropic, though it is much less infective by other routes. After intracerebral inoculation, it produces fatal encephalitis after a short and fixed incubation period of 6–7 days. Negri bodies are usually not demonstrable in the brains of animals dying of fixed virus infection. Fixed virus is used for vaccine production.

2. Chick embryos: Rabies virus can be grown in chick embryos. The usual mode of inoculation is into the yolk sac. Serial propagation in chick embryos has led to the development of attenuated vaccine strains like Flury and Kelev. Strains adapted to duck eggs which give high yields of virus have been used for preparation of inactivated vaccines.

3. Tissue culture: Rabies virus can grow in several

primary and continuous cell cultures such as chick embryo fibroblast, porcine or hamster kidney, but cytopathic effects are not apparent and the yield of virus is low. Fixed virus strains adapted for growth in human diploid cell, chick embryo and vero cell cultures have been used for production of vaccines.

RABIES

Rabies has been recognised from very ancient times as a disease transmitted to man and animals by the bite of 'mad dogs'. The name rabies comes from the Latin word *rabidus*, meaning mad, derived from the Sanskrit root *rabhas*, to do violence. Reference to rabies has been discovered in the Mesopotamian Laws of Eshnunna (circa 1800 B.C.). The disease was traditionally associated with the appearance of the Dog Star Sirius in the 'dog days' of summer when dogs were considered to be prone to spells of 'madness'. The disease in man is called *hydrophobia* because the patient exhibits fear of water, being incapable of drinking though subject to intolerable thirst. Rabies in animals is not called hydrophobia because they do not have this peculiar feature.

The causative agent of rabies had, for centuries, been associated with the saliva of rabid dogs, but it was only in 1804 that Zinke adduced proof by transmitting the disease to normal dogs by inoculation of saliva from rabid dogs. In 1821, Magendie and Breschet infected dogs with saliva *from a human patient, proving the identity of the* agent causing human and animal rabies. In a series of studies dating from 1881, Pasteur established that rabies virus was present in the brains of infected animals. By serial intracerebral passage in rabbits he obtained the fixed virus and demonstrated that dogs could be rendered immune by a series of injections of fixed virus of graded infectivity. This vaccine was prepared by drying for various periods, pieces of spinal cord from rabbits infected with the fixed virus. In July 1885, Joseph Meister, a nine year old lad, severely bitten by a rabid dog and in grave risk of developing rabies, was given a course of 13 inoculations of the infected cord vaccine by Pasteur. The boy survived. This dramatic event marked a milestone in the development of medicine.

Pathogenesis

Man is usually infected by the bite of rabid dogs or other animals. The virus present in the saliva of the animal is deposited in the wound. If untreated, about half of such cases develop rabies. Very rarely, infection can also occur following such nonbite exposures as licks or aerosols containing the virus. Man appears to possess a high degree of natural resistance to rabies. The extent of inapparent or abortive infection with rabies virus in man is not known, but the finding, in one survey, of rabies antibodies in six per cent of veterinarians without any history of antirabic vaccination suggests that it does occur.

The virus appears to multiply in the muscles, connective tissue or nerves at the site of deposition. It penetrates the nerve endings either immediately or after a varying interval and travels in the axoplasm towards the spinal cord and brain. The movement of the virus in the axons is passive, at a speed of about 3 mm per hour. The infection spreads centripetally from the axon to the neuronal bodies, and progressively up the spinal cord through the synapses of neurons. The virus ascends rapidly to the brain where it multiplies. The virus then spreads outwards along the nerve trunks to various parts of *the body including the salivary glands. It multi*plies in the salivary glands and is shed in the saliva. The presence of the virus in the saliva and the irritability and aggression brought on by the encephalitis ensure the transmission and survival of the virus in nature. The virus ultimately reaches virtually every tissue in the body, though the centrifugal dissemination may be interrupted at any stage by death. The virus is almost invariably present in the cornea and the facial skin of patients because of their proximity to the brain. This provides a method for the antemortem diagnosis of human rabies. The virus may also be shed in the milk and urine.

In man the incubation period is usually from 1–3 months, though it may be as short as 10 days or as long as three years. Incubation period is usually short in persons bitten on the face or head, and long in those bitten on the legs. This may be related to the distance the virus has to travel to reach the brain. Incubation period is generally shorter in children than in adults.

The course of the disease in man can be classified into four stages — prodrome, acute neurological phase, coma and death. The onset of the disease is usually initiated by prodromal symptoms such as low grade fever, headache, malaise, fatigue and anorexia. The earliest symptom is often neuritic type of pain or paraesthesia and fasciculation at the site of virus entry. Apprehension, anxiety, agitation, irritability, nervousness, insomnia or depression characterise the prodromal phase, which usually lasts 2–4 days.

The acute neurological phase usually begins with hyperactivity, which is characteristically intermittent, with bouts of bizarre behaviour, agitation or seizures appearing between apparently normal periods. Such hyperactivity may be spontaneous or precipitated by external stimuli. The pathognomonic feature is difficulty in drinking, together with intense thirst. Patients may be able to swallow dry solids, but not liquids. Attempts to drink bring on such painful spasms of the pharynx and larynx producing choking or gagging that patients develop a dread for even the sight or sound of water (hydrophobia). Generalised convulsions follow. Death usually occurs within 1–6 days due to respiratory arrest during convulsions. Some patients progress to paralysis. In rare cases, hyperactivity may not be prominent and paralytic features dominate from the beginning. Such paralytic disease is more common in Latin America and Trinidad after exposure to vampire bat rabies, and in those who have received postexposure vaccination. Death occurs due to respiratory paralysis.

Patients who survive the stage of actue neurological involvement lapse into coma, which may last for hours or months. Death is due to respiratory arrest or other complications.

Some persons exposed to real or imaginary risk of rabies develop a neurotic reaction which has been called lyssaphobia or hydrophobiophobia. Patients present with anxiety, irritability and exaggerated hydrophobia. They are afebrile. Sedation and reassurance are generally all that are called for.

In dogs, the incubation period is usually 3–6 weeks, but it may range from 10 days to a year. The initial signs are an alert, troubled air and a change in disposition with restlessness, snapping at imaginary objects, licking or gnawing at the site of bite. After 2–3 days of this prodromal stage, the disease develops into either the *furious* or *dumb* type of rabies. In furious rabies, which is much commoner, the dog runs amok, biting without provocation and indiscriminately. The lower jaw droops and saliva drools from the mouth. Paralysis, convulsions and death follow. The second type, the dumb rabies is the paralytic form in which the animal lies huddled, unable to feed. The dog may not bite, but attempts to feed it are dangerous. The dumb form is as infectious as the furious type. About 60 per cent of rabid dogs shed the virus in saliva. Rabid dogs usually die in 3–5 days. There have been a number of reports of persons developing rabies after being bitten by apparently healthy dogs. However, in countries like India where stray dogs are so common, it is not always easy to exclude the possibility of such patients having been bitten earlier by other animals. Recently, carrier state in dogs has been confirmed and intermittent salivary shedding of rabies virus over two years demonstrated in a carrier dog. But there is no evidence to indicate that this is anything but a very rare event. The possibility of carrier dogs has not so far altered the recommendations for postexposure treatment. However, when tissue culture vaccines become widely available, the recommendation may change.

Rabies in cats is similar to canine rabies. In cattle and horses, rabies is manifested as irritability, restlessness and unusual aggressiveness, but it is a dead end as they hardly ever bite man or other animals. In wild animals, the chief characteristic

of rabies is the loss of fear of man and other animals. Unprovoked attacks by jackals or other wild animals should be taken as indication of rabies.

Histopathological changes in the brain are minor compared to the clinical evidence of severe neurological damage and the fatal outcome. Vascular congestion, perivascular infiltration and cerebral oedema, with some neuronal destruction are the usual findings. Demyelination in the white matter is common. In the spinal cord, the posterior horns are more severely involved. Some degree of leptomeningitis is common. The greater localisation of the virus in the limbic system, with relative sparing of the neocortex, correlates with the alertness, aggressiveness and loss of natural timidity seen in clinical rabies. The characteristic histopathological feature in rabies is the Intracytoplasmic inclusion body (Negri body) in the neurons, most abundant in the cerebellum and hippocampus.

Laboratory diagnosis

Human rabies: Laboratory diagnosis of human rabies till recently was of little practical importance as death was considered inevitable and no serious attempt at treatment was made, other than heavy sedation. If a patient survived, he was considered not to have had rabies. But now that survival from established rabies has been demonstrated, it is necessary to be able to make laboratory distinction between rabies and other forms of encephalitis, particularly that following antirabic vaccination.

The method most commonly used for diagnosis is demonstration of rabies virus antigen by immunofluorescence. The specimens tested are corneal smears and facial skin biopsy antemortem and brain postmortem. Direct immunofluorescence is done using antirabies serum tagged with fluorescein isothiocyanate. The use of monoclonal antibody instead of crude antiserum makes the test more specific.

Diagnosis may be made postmortem by demonstration of Negri bodies in brain, but they may be absent in some 20 per cent of human cases.

Isolation of the virus by mouse inoculation can be attempted from the brain, CSF, saliva and urine. Chances of isolation are greater early in the disease. A few days after onset, neutralising antibodies appear and the virus can then be isolated only occasionally.

High titre antibodies are present in the CSF in rabies, but not after immunisation. Their demonstration therefore can be used for diagnosis.

Animal rabies: Laboratory diagnosis of rabies in dogs and other biting animals is of great importance in assessing the risk of infection and deciding postexposure treatment. The whole carcass or the severed head of the animal suspected to have died of rabies may be sent to the laboratory. Alternatively, the brain may be removed carefully and two portions, one in 50% glycerol saline and the other in Zenker's fixative, sent for biological test and microscopy, respectively. The portion of brain sent should include the hippocampus and cerebellum as Negri bodies are most abundant there. The following tests are done in the laboratory.

1. Demonstration of rabies virus antigen by immunofluorescence: In experienced hands this is more sensitive than visualisation of Negri bodies, and quite as sensitive as the biological test, with the advantage of immediate results. Examination of salivary glands by immunofluorescence is useful. It may indicate whether the animal was shedding virus in the saliva.

2. Demonstration of inclusion bodies: This is still the method most commonly used as facilities for immunofluorescence and biological test are not available in many laboratories. Impression smears of the brain are stained by Seller's technique (basic fuchsin and methylene blue in methanol), which has the advantage that fixation and staining are done simultaneously. Negri bodies are seen as intracytoplasmic, round or oval, purplish pink structures with characteristic basophilic inner granules. Negri bodies vary in size from 3–27 μ (Fig. 58.2). Other ty

sion bodies may sometimes be seen in the brain in diseases such as canine distemper, but the presence of inner structures in Negri bodies makes differentiation easy. If impression smears are negative the tissue should be sectioned and stained by Giemsa or Mann's method. Failure to find Negri bodies does not exclude the diagnosis of rabies.

3. Isolation of rabies virus (Biological test): The brain tissue suspension is injected intracerebrally into young mice. They die within three weeks, showing numerous Negri bodies in the brain. If several mice are used per specimen, they can be killed serially and brains examined for virus antigen by immunofluorescence. This yields a quicker result.

Prophylaxis

Specific prophylaxis is ideally given before exposure to infection. In animals, this is imperative, but in man, preexposure immunisation was only employed in persons at high risk, such as veterinarians and dog handlers, because neural vaccines carry some risk of serious complications. The introduction of cell culture vaccines which are free from serious complications, has made preexposure immunisation in man safe and feasible. Specific prophylaxis is generally employed after exposure to infection and is therefore called antirabic treatment. This consists of local treatment, antirabic vaccines and hyperimmune serum.

Local treatment: Animal bites deposit the virus in the wounds. Prompt cauterisation of the wounds therefore helps to destroy the virus. The wound should be immediately washed well with soap and water. This is a very important step in the prevention of rabies as soap and water destroy the virus effectively. After washing the soap away completely, the wound is treated with quaternary ammonium compounds (such as cetavlon), tincture or aqueous solution of iodine, or alcohol (40-70 per cent). In severe wounds, antirabic serum may be applied topically and infiltrated around the wound. It is desirable to postpone suturing the wound. Antitetanus measures and antibiotics to prevent sepsis may be used, if necessary.

Fig. 58.2 Negri bodies. Oval or ovoid inclusions in the cytoplasm of nerve cells of the brain of a rabid dog

Antirabic vaccines: Antirabic vaccines fall into two main categories: neural and nonneural. The former are associated with serious risk of neurological complications and are being increasingly replaced by the latter.

NEURAL VACCINES

These consist of suspensions of nervous tissues of animals infected with fixed rabies virus. The earliest was Pasteur's cord vaccine prepared by drying over caustic potash for varying periods pieces of infected rabbit spinal cord. This was replaced by infected brain vaccines, of which there have been several preparations. One of the earliest of these, the Fermi vaccine prepared by treatment with phenol at 22°C contained residual live virus and has therefore been abandoned. The following are the more important:

1. Semple vaccine: This vaccine developed by Semple (1911) at the Central Research Institute, Kasauli, has been the most widely used among antirabic vaccines. It is a 5% suspension of sheep brain infected with fixed virus and inactivated with phenol at 37°C, leaving no residual live virus.

2. *Beta propiolactone (BPL) vaccine*: This is a modification of Semple vaccine in which beta propiolactone is used as the inactivating agent instead of phenol. It is believed to be more antigenic, so that smaller doses are considered adequate. The major antirabic vaccine producing laboratories in India now manufacture BPL vaccine, while the others continue making Semple vaccine.

3. *Infant brain vaccines*: The encephalitogenic factor in brain tissue is a basic protein associated with myelin. It is scanty or absent in nonmyelinated neural tissue of newborn animals. So infant brain vaccines have been developed in order to reduce neurological complications. A vaccine prepared from suckling mouse brain and inactivated by ultraviolet irradiation, beta propiolactone or phenol (Fuenzalida type vaccine) is widely used in South America. Similar vaccines prepared from infant rat brain are in use in the USSR and from suckling rabbit brain in the Netherlands. Occasional cases of neurological reactions have occurred following infant brain vaccines also. Infant brain vaccine is impractical in India due to the very large quantities required.

NONNEURAL VACCINES

I. Egg vaccines 1. Duck egg vaccine: This is fixed virus adapted for growth in duck eggs and inactivated with beta propiolactone. This has been discontinued because of its poor immunogenicity.

2. Live attenuated chick embryo vaccines: Two types of vaccines are available — the Low Egg Passage (LEP) vaccine at 40–50 egg passage level for immunisation of dogs of three months or more, but not for other animals or young pups, and the High Egg Passage (HEP) vaccine at 180 passage level for cattle and cats. The HEP vaccine had been used for preexposure immunisation in man also, but it is not recommended now as it contains live virus.

II. Tissue culture vaccines: The first cell culture vaccine was the human diploid cell strain (HDCS) vaccine developed by Koprowsky, Wiktor and Plotkin. It is a purified and concentrated preparation of fixed rabies virus (Pitman-Moore Strain) grown on human diploid cells (WI 38 or MRC 5) and inactivated with beta propiolactone or tri-n-butyl phosphate. It is highly antigenic and free from serious side effects. Its only disadvantage is its high cost. Other equally effective and more economical cell culture vaccines have been developed. The purified chick embryo cell culture (PCEC) vaccine containing BPL inactivated Flury LEP strain is now widely used. Even more economical is the vero cell vaccine which is expected to become available shortly.

III. Subunit vaccine: The glycoprotein subunit on the virus surface, which is the protective antigen

has been cloned and recombinant vaccines produced. They are still in the experimental stage.

Vaccination schedules: In the advanced countries the neural and duck egg vaccines have been replaced by cell culture vaccines for human and animal use. They are not widely employed in developing countries like India due to the high cost, and neural vaccines continue to be in use. The schedules for both types of vaccines are given below.

Antirabic vaccine should be administered when a person has been bitten, scratched or licked by an animal which cannot be apprehended. When the biting animal can be observed, it should not be destroyed, but should be kept for ten days. The observation period of ten days is recommended because the virus may be present in the saliva 3–4 days before onset of symptoms and the animal usually dies within 5–6 days of developing the disease. If the animal remains healthy after this period, there is no risk of rabies, and vaccine, if already started, may be discontinued. This, of course, does not take into account the rare possibility of carrier state in dogs.

In cases where vaccine is started with the biting animal kept under observation, an alternative recommendation is to stop treatment after five days. The animal is observed for a further five days, vaccine being started again if the animal becomes ill or dies during the period.

The dosage of vaccine depends on the degree of risk to which the patient has been exposed. Accordingly patients are classified as follows:

Class I: Patients in whom the risk is estimated to be slight. These include:

a. Licks, including direct contact with saliva on definitely remembered fresh cuts or abrasions on all parts of the body except the head, face, neck or fingers.
b. Licks on intact mucous membrane or conjunctiva.
c. Bites or scratches which have raised the epidermis but have not drawn blood, on all parts of the body except the head, face, neck or fingers.
d. Consumption of unboiled milk or handling raw flesh of rabid animals.

Class II: Persons who are estimated to be at moderate risk. These include:

a. Licks on definitely remembered fresh cuts or abrasions on the fingers.
b. All bites or scratches on the fingers which are not lacerated, not more than half centimetre long and have not penetrated the true skin.
c. Bites or scratches on all parts of the body except the head, face, neck or fingers which have drawn blood, but excluding bites which have five teeth marks or more, or in which extensive laceration has occurred.

Class III: Persons in whom the risk is estimated to be great. These include:

a. Licks on definitely remembered fresh cuts or abrasions or head, face or neck.
b. All bites or scratches on the head, face or neck.
c. All bites or scratches on the fingers which are lacerated, more than half centimetre long or have penetrated the true skin.
d. All bites penetrating the true skin and drawing blood, when there are five teeth marks or more.
e. All bites on any part of the body causing extensive laceration.
f. All jackal and wolf bites.
g. Any Class II patient who has not received treatment within 14 days of exposure.

The recommended schedule of vaccination for the different classes is as follows:

	Semple vaccine	*BPL vaccine*
Class I	2 ml x 7 days	2 ml x 7 days
Class II	5 ml x 14 days	3 ml x 10 days
Class III	10 ml x 14 days	5 ml x 10 days

(The above schedule for BPL vaccine is as recommended by the Pasteur Institute, Coonoor. The Central Research Institute, Kasauli, recommends a slightly different dosage for its vaccine. The manufacturer's instructions should be followed in every case.)

The immunity following vaccination is expected to last for six months only and any exposure later should receive fresh treatment. The vaccine is administered subcutaneously on the anterior abdominal wall.

Antirabic vaccine may cause certain adverse reactions. These range from minor local reactions to serious neuroparalytic complications. The latter may be of the neuritic type, the dorsolumbar type, the Landry type of ascending paralysis or encephalomyelitis. The aetiology of neurological complication is believed to be immune response to the injected brain tissue resulting in organ specific immunological damage as in experimental allergic encephalomyelitis. These complications usually occur within 1–4 weeks of commencement of vaccination. The incidence of complications varies with different vaccines, one in 2000 to one in 12,000. When such complications are noticed during the course of vaccination, further vaccination should be withheld and the patient started on corticosteroids. If further vaccination is considered imperative, non-neural vaccine should be used. Severe exertion and the use of alcohol during vaccination have been said to increase the risk of neurological reactions.

Cell culture vaccines: For persons at high risk of exposure to rabies infection, preexposure immunisation may be given by three intramuscular doses of 1.0 ml on days 0,7, and 28. An alternative method is to give 0.1 ml intradermally on days 0,7 and 28.

The dosage for postexposure treatment is 1.0 ml IM. on days 0,3,7,14 and 30, with a booster dose on day 90. Intradermal 0.1 ml dose has also been reported as effective. IM injections are to be given in the deltoid, or in children on the anterolateral aspect of the thigh. Gluteal injections are better avoided as they are suspected to be less immunogenic.

In persons who have had preexposure immunisation and subsequently exposed to infection, three doses of vaccine on days 0,3 and 7 are adequate. Antirabies serum or globulin should not be given in such cases.

Antirabic serum: This is prepared by hyperimmunisation of horses. This is a useful adjunct to vaccine in cases of severe bites and should be given as early as possible after exposure, and in any case within five days, after which it may not be beneficial. The dose recommended is 40 IU per kg body weight. The usual precautions against hypersensitivity to horse serum should be taken. Human antirabies immunoglobulin has been prepared to avoid risk of hypersensitivity. Its recommended dose in 10 IU/kg. Serum should not be given to persons who have had prior active immunisation. As administration of serum depresses the active immune response to some extent, it is necessary in such cases to give booster doses of the vaccine after the regular 14-day course is over. Booster injections are recommended 10,20 and 90 days after the last daily dose of vaccine for neural vaccines and a single dose on day 90 for cell culture vaccine.

It is not always easy to make a decision whether or not to administer antirable vaccine in cases of doubtful exposure to infection. Local treatment should be applied promptly in all cases. Serum is reserved for the cases adjuged to be at high risk. As the neural vaccines available carry a real risk of serious complications, they should not be used indiscriminately, but only when, in the judgement of the physician, there is some risk of infection. It is important to remember that while post-exposure treatment affords some protection against rabies, it is by no means absolute. There occur several cases of 'vaccine failures', where persons given a full course of vaccine develop rabies. Estimates of protection afforded by the vaccine have varied widely. Pasteur Institute, Coonoor, reported that among persons bitten by rabid dogs proved to be infective, the mortality rates in those who received the complete course of vaccine and those who did not were 3.5 per cent and 68 per cent, respectively.

Vaccines for animals: Neural vaccines are unsatisfactory as they are not adequately immunogenic, need multiple doses and have to be repeated every six months. Flury vaccines

gave immunity for three years, but were of uncertain efficacy as the viability of the vaccine virus at the time of administration could not be ensured. Cell culture vaccines containing BPL inactivated virus adsorbed on aluminium hydroxide are now used for animal immunisation. They are highly immunogenic after a single 1.0 ml, IM dose. Booster doses need be given only at 1–3 years intervals. The earliest age for vaccination of pups born to nonimmune mothers is one month and to those of immunised mothers three months. Rabies vaccines may be given separately or as combined multiple vaccines for immunisation against other common canine infections also.

Treatment: Till recently, rabies was considered to be invariably fatal and no serious attempt at treatment was made, apart from sedation. It has now been demonstrated that complete recovery can occur from established rabies, with intensive supportive care and management of complications. No specific antirabies agent is available. It has been suggested that in nonimmunised patients, administration of hyperimmune serum may be beneficial. Interferon has also been used in treatment.

Human rabies is a dead end. Direct man to man transmission of rabies has not been recorded, though the virus is present in the saliva of patients. Therefore there is no danger in examining or nursing hydrophobia patients provided suitable precautions are taken. An unusual mode of transmission of rabies has occurred in some recipients of corneal grafts. The donors had died of unsuspected rabies and the infection was transmitted through the cornea.

Epidemiology: Most cases of human rabies follow dog bites, but in endemic areas almost any animal can transmit rabies. In India, antirabic treatment is indicated following the bite of any animal except the rat. Where domestic rabies has been controlled, as in the USA, the majority of infections are due to bites by wild animals.

The primary source of rabies virus in nature seems to be in the mustelids and viverrids, the ermine in the northern coniferous forests, the skunk, mink and weasel in North America, the mottled pole cat in the USSR, the civet and pole cat in Africa and the mongoose in Asia. Rabies virus has been isolated repeatedly from the brain and salivary glands of apparently healthy wild rodents. The virus survives in this reservoir population by achieving a state of latency with occasional activation such that only a small proportion of them will be shedding the virus at any one time. From the reservoir species, wild vectors such as foxes, wolves and jackals acquire the infection and occasionally epizootics occur in these species. A smouldering epizootic of rabies in the red fox in Europe has been spreading westwards steadily from Poland to France during the last two decades. Carnivorous animals may acquire the infection by eating carcasses containing the virus. From these species the disease spreads to dogs and other domestic animals.

Another natural cycle of rabies concerns bats. A fatal paralytic disease of cattle and man was noticed in Trinidad in 1925. This was identified as rabies only years later when rabies virus was recovered from a human case mistaken for poliomyelitis. The disease was shown to be transmitted by vampire bats that sweep down on their prey at night. Vampire bat rabies occurs in Mexico, Central and South America and takes a heavy toll of cattle. Vampire bats may shed rabies virus as symptomless carriers over a period of several months.

Rabies occurs in insectivorous and frugivorous bats also. While in canines rabies is neurotropic, in bats the virus is primarily adapted to the respiratory tract. Man may be infected by aerosols if he enters caves where infected bats colonise. Pneumotropic rabies virus strains have been obtained from bats. Bat rabies is largely confined to the Americas. A few strains of rabies virus have been isolated from bats in Europe, but their epidemiological significance is not known.

Rabies is endemic in India. It has been estimated that more than 15,000 people die of rabies in India every year and more than 700,000 receive antirabies vaccine. Human rabies can be checked

by control of rabies in domestic animals, by registration, licensing and vaccination of pets and destruction of stray animals. But rabies can be eliminated only if the wild vectors such as jackals and foxes, and the reservoir mustelids and viverrids are controlled. Rabies has been eliminated from islands like Britain and Japan by rigid quarantine. Australia which has no native mustelid or viverrid population has no rabies. Eradication of rabies from countries like India with abundant wildlife may not be practicable.

RABIES-RELATED VIRUSES

Rabies virus was for long considered to be a uniquely distinct serological entity. This view has been challenged by the discovery of a number of viruses in Africa, which show varying degrees of antigenic relationship with rabies virus. Some of them have been associated with human disease. This introduces new problems concerning the transmission, diagnosis and prophylaxis of rabies. Rabies-related viruses include the Lagos bat virus, Nigerian horse virus, Mokola virus, Duvenhage virus, Obodhiang virus, Kotonkan virus, Oulo-fato virus, Bolivar virus and the Czechoslovakian rodent viruses.

Lagos bat virus was isolated in 1956 from pooled brains of frugivorous bats from Lagos Island, Nigeria. It causes a rabies-like illness following intracerebral inoculation. Negri bodies are found in infected monkey brain, but not in mice or dogs.

Nigerian horse virus was isolated in 1958 in Ibadan, Nigeria, from the brain of a horse that died of 'staggers', a rabies-like illness.

Mokola virus was isolated in 1968 from shrews captured near Ibadan. Later, it was also recovered from two children with central nervous system disease, one of whom died. A case of laboratory infection with the virus occurred in a person possessing high titre neutralising antibody to rabies virus. It resembles Lagos bat virus in pathogenicity.

Duvenhage virus was isolated from the brain of a man who died in South Africa of clinical rabies after being bitten by a bat. A related virus has been reported in bats from Eastern Europe.

Obodhiang and Kotonkan viruses were isolated from arthropods, from Mansonia mosquitoes in Sudan and from culicoides midges in Ibadan, respectively.

Oulo-fato is a mild paralytic disease of dogs in West Africa. The causative agent had been considered to be rabies virus, but serological differences have now been identified between them. Bolivar virus was isolated from cattle in Venezuela. Several strains of rabies-like viruses have been recovered from rodents in Czechoslovakia.

These viruses show varying degrees of cross reaction with rabies virus in complement fixation and immunofluorescence tests, but appear to be distinct by neutralisation test. Some of them can cause human infection, which does not appear to be influenced by antibodies against rabies. If, in future, they are found to be more widespread and to cause human disease more frequently, the strategy of rabies prophylaxis may have to be revised.

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59 Hepatitis Viruses

The term 'viral hepatitis' refers to a primary infection of the liver by hepatitis virus type A or type B, or by other hepatitis viruses as yet not characterised and referred to as 'non-A: non-B' hepatitis viruses. Hepatitis may occur incidentally during the course of infection with many other viruses, such as yellow fever, Lassa fever, Marburg, EB virus, cytomegalovirus, herpes simplex, rubella or coxsackievirus, but this is not included under the category of viral hepatitis.

By epidemiological and clinical criteria, two types of viral hepatitis had been recognised. One type occurs either sporadically or in epidemics, affecting mainly children and young adults, and apparently transmitted by the faecal-oral route. This was called infective or infectious hepatitis. It is now known as type A hepatitis. A second type of viral hepatitis is transmitted mainly by inoculation, and was originally observed in persons receiving serum inoculation or blood transfusion. This had been known by several names, such as homologous serum jaundice, or serum hepatitis. It is currently called type B hepatitis.

For a time it was believed that all viral hepatitis was caused by either of the two hepatitis viruses, type A accounting for all infectious hepatitis and type B for all post transfusion or serum hepatitis. But with the development and application of techniques for the detection of infection with hepatitis type A and B viruses, it became apparent that in many cases of infectious hepatitis and post transfusion hepatitis, no evidence could be found of infection with either type A or type B hepatitis viruses. It therefore became evident that the clinical syndrome of type A or B hepatitis could be produced also by other uncharacterised viral agent or agents. The term 'non-A: non-B' hepatitis has been applied to this group.

TYPE A HEPATITIS
(Infectious Hepatitis)

Hepatitis type A is a subacute disease of global distribution occurring mainly in children and young adults. The virus enters the body by ingestion and multiplies in the intestinal epithelium before it reaches the liver by haematogenous spread. It is shed in the faeces during the late incubation period and the prodromal phase of the illness, but only infrequently afterwards. Once jaundice develops, it is rarely detectable in faeces. The virus is present in the blood for a brief period during the pre-icteric stage, but usually disappears when jaundice sets in. It may be occasionally present in the urine and saliva.

Clinical features: The incubation period is 2–6 weeks. The clinical disease consists of two stages, the prodromal or pre-icteric stage and the icteric stage. The onset may be acute or insidious with fever, malaise, anorexia, nausea, vomiting and liver tenderness. These usually subside with the onset of jaundice. Recovery is slow, over a period of 4–6 weeks. Occasional cases develop a rapidly fatal fulminant hepatitis. The disease is milder in children, in whom most infections are anicteric. Mortality is low, ranging from 0.1 to 1 per cent, with most of the deaths occurring in adults.

Epidemiology : The disease is worldwide in distribution. It is more common in children and young adults. In temperate regions, it shows an autumn-winter predilection, but in tropics no seasonal distribution is evident. In India the disease tends to be associated with periods of heavy rainfall.

Faecal virus constitutes the most important source of infection. Chronic intestinal carriage is extremely rare and carriers do not appear to be epidemiologically important. The virus is maintained in nature by serial faecal-oral transfer, most commonly by person to person contact. Infection occurs readily under conditions of poor sanitation and overcrowding. Several food borne epidemics have been described in which the source has been attributed to virus shed by food handlers during the incubation period of the disease. Ingestion of shell fish grown in polluted waters has also caused the disease. Water borne epidemics have been documented. The virus can be destroyed by adequate chlorination of water (total residual chlorine of one part per million and free residual chlorine of 0.4 ppm;, but chlorination may not be effective in the presence of organic pollution).

Blood of patients in the viraemic phase is infectious and parenteral transmission may occur very rarely. But chronic viraemic carriers do not occur and parenteral transmission is of little epidemiological significance. Transplacental transfer of the virus has not been demonstrated. Instances have been recorded of transmission of the disease from chimpanzees to persons in contact with them. The animals are believed to have acquired the infection from man. There is no evidence to suggest any extrahuman source of the virus in nature.

The epidemiology of type A hepatitis resembles that of poliomyelitis. In the developing countries, infection is acquired in childhood and by the age of ten, 90 per cent of the population possess antibody to the virus. In India, type A hepatitis is the most common cause of acute hepatitis in children, but is much less frequent in adults. In affluent countries, the incidence of the disease has been declining with an upward shift in the age group affected.

Hepatitis A virus (HAV)

In 1973, Feinstone and coworkers, using immunoelectronmicroscopy demonstrated this virus in the faeces of experimentally infected human volunteers. Experimental infection can be produced in chimpanzees and South American marmoset monkeys. The virus is grown with difficulty in some human and simian cell cultures. The virus has been cloned.

HAV is a 27 mm nonenveloped RNA virus belonging to the picornavirus family. It has been designated 'enterovirus 72'. Only one serotype of the virus exists.

The virus is relatively resistant to inactivation by heat at 60°C for one hour, ether and acid at pH 3, but inactivated by 1:4000 formaldehyde at 37°C for 72 hours and by chlorine at 1 ppm for 30 minutes. It is not affected by nonionic detergents. It stands prolonged storage at 4°C or colder temperatures.

Laboratory diagnosis: Aetiological diagnosis of type A hepatitis is now possible by demonstration of the virus or its antibody, but the tests are available only in a few specialised laboratories. The techniques employed are immune electron microscopy, complement fixation, immune adherence, haemagglutination, radioimmunoassay and enzyme immunoassay. Virus can be demonstrated in faecal extracts during the late incubation period and preicteric stage, but seldom after jaundice develops. Antibodies appear early in the course of the disease. Rise in titre of antibodies may be demonstrated in paired sera. When this is not possible, detection of IgM antibody in serum indicates recent infection. IgG antibody may indicate either recent or remote infection (Fig. 59.1).

Prophylaxis : As the disease is transmitted by the faecal-oral route, general prophylaxis consists of improved sanitary practices and the prevention

of faecal contamination of food and water. Passive prophylaxis with human gamma globulin is effective. Normal pooled human immunoglobulin (16% solution in a dose of 0.02 to 0.12 ml per kg body weight I.M) before exposure to the virus or in the early incubation period may prevent or attenuate a clinical illness, while not necessarily preventing virus excretion. One attack of the disease is believed to confer immunity to the homologous infection, but not to type B or non A: non B hepatitis. As only one serotype of hepatitis A virus exists, it may be possible to develop a vaccine when suitable methods become available for cultivation of the virus.

TYPE B HEPATITIS
(Serum Hepatitis)

Type B hepatitis was originally considered to be an unusual disease transmitted exclusively by the parenteral route, and by iatrogenic procedures such as blood transfusion or injection of serum or other blood products. Its natural history has only recently become clear. It is now known to be transmitted also by many natural mechanisms that transfer minute quantities of blood or tissue fluid between persons.

Clinical features : The incubation period is long, 2–6 months, as against the shorter incubation period of type A hepatitis (2–6 weeks). The clinical disease is similar to type A hepatitis, but is usually more severe and protracted. The onset is insidious and fever is not prominent. Extrahepatic manifestations are common. These consist mainly of rash, arthralgia, polyarteritis nodosa and glomerulonephritis and are believed to result from antigen-antibody combination *in vivo*. Some patients progress to chronic active hepatitis and cirrhosis. Primary hepatocellular carcinoma has been shown to be a late consequence of HBV infection. The viral DNA is found integrated into the genome of malignant hepatocytes. The case fatality rate is from 0.5 to 2 per cent, but higher mortality has been observed in post transfusion hepatitis.

Epidemiology : The disease occurs throughout the world. It has no seasonal distribution. The incidence is more in adults than in children and in urban than in rural areas. The virus is maintained in nature by chronic viraemic carriers, who are estimated to number over 200 millions, some 35 millions of whom are in India. About 5–10 per cent of infected persons become carriers. Persons possessing hepatitis B surface antigen in blood for more than six months are known as chronic or persistent carriers. Subclinical infections are more likely to lead to chronic carriage than acute hepatitis. The reported prevalence of carriers in different populations varies very widely, from 0.1 per cent in the advanced countries to 20 per cent

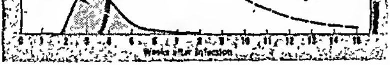

Fig. 59.1 Typical course of hepatitis A.

in the developing nations. Carrier rate is higher in tropical than in temperate regions, and in males than in females. Though acute and chronic infection can be established experimentally in chimpanzees, no natural animal reservoir is known for the virus.

Transmission is mainly by the percutaneous route. Besides blood transfusion, a number of therapeutic, prophylactic and diagnostic procedures can convey the infection. The virus is highly infectious and very minute amounts of some carrier sera (as little as 0.00001 ml) can transmit the disease. Therefore, any procedure that can convey traces of blood or serum from one to another can serve to spread the infection. Tattooing, acupuncture, sharing of razors, sexual intercourse and even kissing can transmit the virus. Dentists and barbers may infect their clients. The disease is particularly common among drug addicts, prostitutes and male homosexuals. Mechanical transmission of virus by blood sucking insects has been suggested. While the virus has been demonstrated in wild caught mosquitoes and bed bugs, their role in transmission is not proved. Infection by ingestion has been suspected, but requires confirmation. Experimental infection by feeding is possible, but relatively large doses are needed. There are reports of the virus being present in various body fluids and excretions such as urine, saliva, milk, semen, bile and faeces. Transplacental transmission has been documented and congenital infection has been reported to be more common in the tropics. Transmission among members of a family appears to be due to shared articles.

Certain groups and occupations carry a high risk of developing infection. These include medical and paramedical personnel, staff of blood banks and haemodialysis units, laboratory workers and staff of institutions for the mentally retarded.

Hepatitis B virus (HBV)

In 1965, Blumberg and coworkers reported a protein antigen in the serum of an Australian aborigine, which gave a clearly defined line of precipitation with sera from two haemophiliacs who had received multiple transfusions. This antigen was called *Australia antigen*. By 1968, the Australia antigen was shown to be associated with serum hepatitis. It was subsequently shown to be the surface component of hepatitis B virus (HBsAg).

Examined under the electron microscope, sera from type B hepatitis patients show three types of particles (Fig. 59.2). The most abundant form is a spherical particle, approximately 22 nm in diameter. The second type of particle is tubular, with a diameter of 22 nm and of varying length. These two types of particles are antigenically identical. They represent the 'Australia antigen' and are surface subunits of the hepatitis B virus, which are produced in great excess. The third type of particle, which is the least numerous, is a double shelled spherical structure, 42 nm in diameter. This particle is the complete hepatitis B virus. It was first described by Dane and his colleagues in 1970 and was therefore known as the Dane particle. The virion consists of a core of 27 nm diameter, with a 2 nm thick shell and an outer coat or envelope 7 nm thick. The core possesses icosahed-

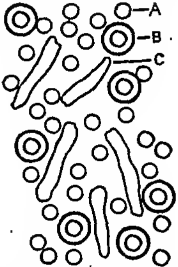

Fig. 59.2. Different types of particles seen in hepatitis type B serum. A. spherical 22 nm particle, B double-shelled 42 nm particle (Dane particle), C tubular 22 nm particle.

ral symmetry. Hepatitis B virus is a DNA virus containing double stranded DNA and a DNA dependent DNA polymerase. The inner core of the virion is antigenically distinct from the outer envelope. The latter is serologically identical with the 'Australia antigen'. (Fig. 59.3 and Fig. 59.4)

Hepatitis B virus has been classified within a new family of viruses called 'Hepadnaviruses' which includes the related viruses found in the ground squirrel, Woodchuck, prairie dog and Pekin duck. Hepadnaviruses can integrate with host cell DNA, leading to persistent infection or malignant transformation.

A variety of names had been used to describe the different antigens and antibodies that characterise viral hepatitis. The terminology has been standardised and the terms used currently are given below.

HAV	Hepatitis A virus.
anti-HAV	Antibody to hepatitis A virus.
HBV	Hepatitis B virus. A 42 nm double shelled virus, originally known as the Dane particle.
HBsAg	Hepatitis B surface antigen. The antigen found on the surface of the virus as well as on the free 22 nm spherical and tubular particles seen in hepatitis B sera. Formerly known as Australia antigen.
HBcAg	Hepatitis B core antigen, found on the 27 nm core of the virus.
HBeAg	Hepatitis B e antigen associated with the core of the virus and also with infectivity of carrier blood.
anti-HBs	Antibody to hepatitis B surface antigen.
anti-HBc	Antibody to hepatitis B core antigen.
anti-HBe	Antibody to hepatitis B e antigen.

HBsAg exhibits antigenic diversity. It consists of at least three different antigenic components: a group specific antigen *a* and two pairs of type specific antigens *d-y* and *w-r*, only one member of each pair being present at a time. HBsAg can thus be divided into four major antigenic subtypes: *adw*, *adr*, *ayw* and *ayr*. A number of other antigenic subtypes have been described, based on complex permutations of these subdeterminants and their variants. Additional surface antigenic reactivities, such as *q,x,f,t,j,n* and *g* have been identified, but they, have not been characterised as yet.

HBsAg subtypes show a distinct geographical distribution. The subtype *ayw* is predominant in a broad geographical zone extending from West Asia, through Iran and Pakistan to India. Subtype *adw* is predominant in Europe and the Americas, while *adr* is prevalent in South East Asia and the Far East. Subtype *ayr* is extremely rare.

Hepatitis B carriers: Based on serological markers, hepatitis B carriers are classified into two categories. Those who have HBeAg in blood and are in the early stages of carrier state are very highly infectious. Very minute amounts of serum or blood from such carriers can transmit the infection. They have high titres of HBsAg and

Fig 59.3 Electron micrograph of HBV — Pooled Plasma (Total magnification 11,4000)
(Courtesy: National Institute of Virology, Pune)

DNA polymerase in their blood and have mildly raised serum transaminase levels. HBV may be demonstrable in their blood. They are known as *super carriers*. Super carrier mothers very commonly infect their infants and such infants in turn become carriers. The term *simple carrier* is applied to the more common type of carrier who has no HBeAg and a low level of HBsAg in blood. HBV and DNA polymerase are absent. Simple carriers transmit the infection only when large volumes of blood or serum are transferred, as in blood transfusion. Simple carriers represent the later stages of the carrier state.

Immune response: The immune response in hepatitis B involves three antigenic systems — HBs, HBc and HBe antigens. Both humoral and cellular responses occur during the infection.

Antibody to HBsAg is associated with resistance to infection. Antibody to HBc is not protective, but appears to be related to the amount and duration of replication of the virus. The highest titres of anti-HBe are found in persistent HBsAg carriers. Antibody to HBeAg is seen in sera of carriers with low infectivity. The antibody response during the disease is not entirely beneficial to the host; it appears to contribute to pathogenicity, particularly in fulminant hepatitis. Circulating immune complexes are frequent and are believed to be responsible for the extrahepatic complications associated with the disease.

Cell mediated immunity to the surface antigen as measured by leucocyte migration inhibition develops during the acute phase of the illness and disappears soon after recovery. It is negative in asymptomatic carriers. However, it persists in patients who progress to chronic active hepatitis. Cell mediated immunity may be involved in terminating the infection and, under certain circumstances, in promoting hepatocellular damage and in the genesis of hepatic autoimmunity. A normal T cell function is considered to be a prerequisite for the self-limited course of hepatitis, whereas a defective function or its absence may favour the development of chronic liver damage and the asymptomatic carrier state, respectively.

Laboratory methods for detection of hepatitis B antigens and antibodies: A variety of serological techniques are available for the detection of hepatitis B antigens and antibodies, which serve to diagnose acute and chronic infections with the virus, and its carrier state.

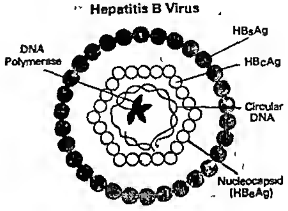

Fig. 59.4 Hepatitis B virus structure.

HBsAg is recognised as a specific marker for infection with the virus. It becomes detectable in circulation about a month after exposure to infection and several weeks before elevation of transaminase levels and appearance of jaundice. Peak levels of HBsAg are seen in the pre-icteric phase of the disease. HBsAg disappears with recovery from clinical disease in most patients, but in a small proportion, it persists for years. These are the carriers. Antibody to HBsAg appears within weeks after the disappearance of HBsAg and persists for years. Immunity to reinfection appears to be related to the presence of anti-HBs.

HBcAg is not detectable in the serum of patients but can be demonstrated in liver cells by immunofluorescence. Antibody to the core antigen usually appears in the pre-icteric phase and persists for months.

A third system associated with type B hepatitis

is the *e* antigen and its antibody. HBeAg is a hidden antigenic component of the virus core, probably a monomeric form or breakdown product of HBcAg. It appears in the serum at the same time as HBsAg, but in most cases it disappears within a few weeks. Its disappearance is followed by the appearance of anti-HBe in serum. This lasts for several months. HBeAg is believed to correlate with the number of viral particles and the degree of infectivity of carrier sera. Sera containing HBeAg are believed to be highly infectious and those with anti-HBe of little infectivity. The presence of HBeAg is also thought to be an adverse prognostic sign as regards the severity and course of the disease. Immunodiffusion methods are employed for the detection of HBeAg and its antibody.

Viral DNA polymerase can be detected transiently in serum during the pre-icteric phase of the illness (Fig. 59.5).

The methods used for the detection of hepatitis antigens and antibodies, listed according to their sensitivity are shown in Table 59.1. Serological examination helps in determining the stage of hepatitis B infection and its carrier state (Table 59.2).

Delta agent: In 1977 Italian workers identified a new antigen-delta in liver cells and blood of some HBsAg carriers. The delta agent is a defective virus that needs HBV as a helper for replication. Its genome consists of low molecular weight RNA. In blood it occurs as a 35–37 nm particle with the cental RNA core surrounded by an HBsAg envelope. Simultaneous infection with HBV and delta leads to biphasic hepatitis with

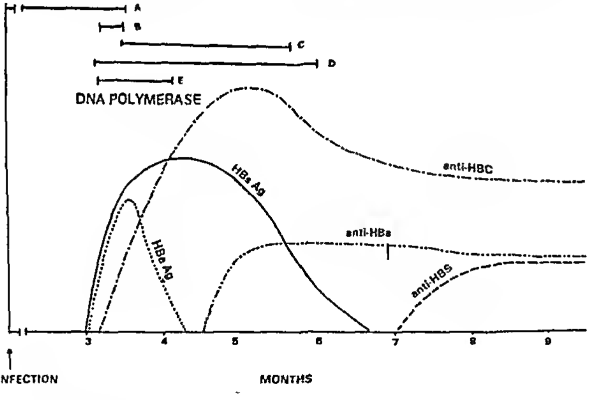

Fig 59 5 The development of serological markers in acute HBV infection A. Incubation B Prodrome. C Symptoms D Transaminases E HBV and DNA polymerase

TABLE 59.1

Methods for detection of HBsAg and anti HBs

Sensitivity	Tests
Low	Immunodiffusion
Moderate	Complement fixation Counterimmunoelectrophoresis Reversed passive latex agglutination Passive haemagglutination Immune adherence haemagglutination Immune electron microscopy
High	Radioimmunoassay Radioimmunoprecipitation Enzyme immunoassay

two transaminase peaks separated by 2–3 weeks. Superinfection with delta in HBsAg carriers may lead to acute and progressive liver injury. Delta agent has been found worldwide.

Prophylaxis. Apart from sporadic cases in the community, hepatitis B infection occurs mainly following blood transfusion, injection of blood products, unsterile invasive procedures, in drug addicts, in male homosexuals, in medical and laboratory personnel handling infected patients and blood, in inmates of dialysis units and mental hospitals and in infants of carrier mothers. Outbreaks have occurred in hospital personnel. General preventive measures include health education, improvement of personal hygiene and strict attention to sterility. An important preventive measure is the screening for HBsAg and HBeAg in blood donors. This has become standard practice in well run blood banks.

Passive immunisation may be employed following any acute exposure to hepatitis B infection. Human serum immune globulin has been found to give some degree of protection. The extent of protection would depend on the titre of anti-HBs in the preparation. It is preferable to use antihepatitis B immune globulin prepared from sera with high titres of anti-HBs. Though immunoglobulin preparations are expected to be free from the risk of transmitting hepatitis B, their use has, on occasions, caused outbreaks of the disease.

Active immunisation can be achieved with heat inactivated human carrier plasma as the source of

TABLE 59.2

Interpretation of serological tests in Hepatitis B

HBsAg	HBeAg	anti-HBs	anti-HBc	anti-HBe	Interpretation
+	+	–	–	–	Late incubation period or early stage of hepatitis. Highly infectious
+	+	–	+	–	Acute hepatitis; or super carrier. Highly infectious
+	–	–	+	–	Simple carrier; infectious
+	–	–	+	+	Acute hepatitis later stage; or carrier with low infectivity
–	–	+	+	+	Late convalescence

HBsAg which is the protective antigen. In super carriers, 1.0 ml of plasma may contain about one trillion HBsAg particles. After purificatioo aod concentration, any possible residual virus is killed with 1:4000 formaldehyde. This vaccine has alum as adjuvant. Field trials showed protection of over 90 per cent. The vaccine is now available for use in high risk groups. The recommended schedule is three doses of one ml given I.M., the second dose a month after the first, and the third six months later. The high cost and limited availability are the only disadvantages. The vaccine is recommended for babies born of HBsAg positive motbers, family and sexual contacts of carriers, patients and staff of haemodialysis units, staff of high risk places such as blood banks, serology laboratories and dental clinics, inmates and staff of mental hospitals, intravenous drug abusers, male bomosexuals and prostitutes.

By cloning the virus DNA in *E. coli*, yeast and vaccinia virus, a number of recombinant vaccines have been produced experimentally. Recombinant HBsAg vaccine produced in yeast induces good antibody response in human volunteers.

NON A: NON B HEPATITIS (NANB hepatitis)

The term refers to viral hepatitis resembling type A or type B clinically and epidemiologically but not caused by either of these viruses. This is not a single entity, but a group of distinct infections by different agents. Epidemiologically at least three types have been recognised.

The transfusion-associated type is seen in recipients of blood transfusion. In the U.S.A. and other countries where effective screening of donors of HBsAg is practised, almost all post transfusion

TABLE 59.3
Comparison of hepatitis types A and B

	Type A	Type B
1 Incubation period	2-6 weeks	2-6 months
2 Onset	Usually acute	Insidious
3 Fever less than 38°C	Usual	Rare
4 Extra-hepatic lesions	Absent	Frequent
5 Age incidence	Children and young adults	All ages; commoner in adults
6 Seasonal distribution	Autumn-winter; Postmonsoon in India	All the year round
7 Secondary cases	Common	Rare
8 Homologous immunity	Present	Present
9 Heterologous immunity	Absent	Absent
10 'Australia antigen' in blood	Absent	Present
11 Raised serum IgM	Common	Rare
12 Raised serum transaminase	Few days	Many weeks
13 Viraemia	Brief	Prolonged
14 Virus in faeces.	Present early	Absent
15 Chronic carriers	Absent	Present
16 Usual mode of infection	Oral	Parenteral
17 Virus size	27 nm	42 nm
18 Virus nucleic acid	RNA	DNA
19 Virus stability		
(a) Heat 60°C for 60 mins	Survives	Survives
100°C for 5 mins.	Inactivated	Inactivated
(b) Formaldehyde 1:4000	Inactivated	Inactivated

Update on Viral Hepatitis

Type	A	B	C	D	E
Previous designation	Infectious hepatitis	Serum hepatitis	(Part of NonA:NonB hepatitis)	Delta hepatitis	Enterically transmitted NonA:NonB hepatitis
Virus	HAV Nonenveloped RNA virus-Picornavirus-Enterovirus Type 72	HBV Enveloped DNA virus	HCV Enveloped RNA virus Resembles Flavivirus	HDV Defective virus with circular RNA genome resembling plant satellite viruses and viroids	HEV Nonenveloped RNA virus. Resembles calicivirus
Common modes of transmission	Faecal-oral	Percutaneous-Blood and blood products; tissue fluids; drug addicts; sexual contact; vertical transmission.	Blood and blood products	Blood and blood products	Faecal-oral
Incubation period	2-6 weeks	2-6 months	6-8 weeks	Variable	2-9 weeks
Common age group affected	Children	Any age	Any age	Any age	Adults
Mortality	Low	High	Moderate	High	Low, but high in pregnant women
Risk of liver cancer	Nil	Present	Present	Nil	Nil
Geographical distribution	Worldwide	Worldwide	USA, Europe, Japan (Inadequate data from other countries)	Patchy distribution common in Mediterranean, Central America, Rare in India, S.E. Asia	Developing world—Indian subcontinent, Central and S.E. Asia, Central and South America, North Africa

most common

hepatitis is of this type. The incubation period is about two months. The acute illness is mild and may last many months with fluctuations in serum transaminase levels. In some, the disease becomes chronic and may progress to cirrhosis.

There is also another type related to repeated administration of blood and blood products. The incubation period is only 1–2 weeks. This has a high chronicity rate.

The above two types of NANB hepatitis can be transmitted to chimpanzees and human volunteers, but specific viruses and antigens have not been characterised. There is some evidence that one type may be caused by a retrovirus.

The third type is transmitted by the faecal-oral route, either directly from person to person, or through water. This has been termed 'enterically transmitted' or 'epidemic NANB (ENANB). The disease resembles type A hepatitis. Rarely, it may run a fulminant course, particularly in pregnant women. This is the ~~most common type of~~ viral hepatitis in India, having caused several ~~water borne epidemics.~~ Adults are more commonly affected than children in outbreaks. The mortality rate is low (0.1–1 per cent) but in pregnant women it may be much higher (10–20 per cent). A 27 nm virus has been observed by IEM in faeces of patients in the early acute phase of the illness. The infection has been transmitted to human volunteers by feeding such faecal extracts.

The largest recorded epidemic of viral hepatitis which occurred in Delhi in the winter of 1955–56 involving over 30,000 cases within six weeks is now believed to have been of this type. The epidemic was caused by sewage contamination of the city water supply. The disease is also prevalent in South and South East Asia, North Africa and Mexico.

Till their causative viruses or specific antigens are characterised, the diagnosis of NANB hepatitis rests on the exclusion of infection with type A and B viruses.

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60 Miscellaneous Viruses

PAPOVAVIRUSES

The term 'Papova' is a sigla indicating the names of viruses included in this group: *pa*pilloma virus of man and rabbits, *po*lyoma virus of mice and *va*cuolating virus of monkeys.

They are small, nonenveloped, icosahedral viruses containing DNA. Almost all members of this group can cause tumours. Most of the naturally occurring papovavirus tumours are benign, but some such as rabbit papilloma are potentially malignant Polyoma and the vacuolating virus SV 40 produce malignant tumours when inoculated into newborn mice or hamsters. These viruses have been widely employed in the study of viral oncogenesis.

Polyoma virus often causes latent infection in laboratory mice. But when inoculated into newborn mice, it produces a wide variety of malignant tumours. Hence the name *'polyoma'*.

Simian vacuolating virus (SV 40) was isolated from uninoculated rhesus and cynomolgus monkey kidney tissue cultures. The virus did not produce any cytopathic effects in the original cultures, but when fluid from such cultures was inoculated into kidney cell cultures from other simian species (African green or grivet monkey), cytopathic effects occurred, consisting of prominent cytoplasmic vacuolation. SV 40 is oncogenic in newborn hamsters. Its only medical importance is that, because of its oncogenic potential, live viral vaccines should be manufactured only in monkey kidney tissue cultures tested and found free from SV 40 infection.

The common human disease produced by a papovavirus is papilloma or wart. Human papilloma virus is host specific, infecting only man and growing only in organ cultures of human skin. Human warts (verruca vulgaris) usually occur in crops on localised areas of exposed skin.

A special type of wart is condyloma acuminatum or genital wart which is a more moist, soft, pedunculated wart found on the external genitalia. This may be transmitted venereally and may occasionally turn malignant. Though warts in different sites may differ in their appearance, they are caused by the same virus, the morphology being determined by the location. Warts are transmitted by contact and may spread by scratching or autoinoculation. Warts may be transmitted to human volunteers by intradermal inoculation of filtrates from lesions. Antibody (IgM) can be demonstrated in sera of patients by gel diffusion or virus agglutination, but it does not seem to be related to regression of lesions. Most lesions regress spontaneously.

Human papovaviruses have been isolated from a number of patients with impaired immunity. The JC virus was isolated in 1971 from the brain of a patient with Hodgkin's disease and progressive multifocal leukoencephalopathy (PML). Isolation of similar strains from PML cases has been reported from the USA, England and France. JC virus grows only in human fetal glial cell cultures. It is oncogenic, producing malignant gliomas following intracerebral inoculation in newborn hamsters.

Another human papovavirus is the BK virus, isolated in 1971 from the urine of a patient with kidney transplant. Several similar isolates have

been recovered from other kidney transplant patients. BK virus differs from JC virus in being able to grow in a wide range of primary and continuous cell cultures. It is less oncogenic. Both JC and BK viruses agglutinate guinea pig and chicken erythrocytes.

Antibodies to JC and BK viruses are present widely in human sera, being found in about 75 per cent of adults. It is believed that primary infection with these papovaviruses occurs commonly in childhood. The brain and renal tract are the two sites where virus multiplication has been demonstrated. The infection is ordinarily asymptomatic, but the virus may remain latent. If the immune system is impaired in later life following disease or transplantation, the virus may be reactivated, leading to PML or renal disease.

An aetiological relationship has been established between cancer of the uterine cervix and human papilloma viruses types 16 and 18.

PARVOVIRUS

These are very small (about 20 nm) viruses with a single stranded DNA genome. A parvovirus (B19) originally discovered in the blood of symptomless donors has, in 1983, been shown to be associated with aplastic crises in sickle cell and other haemolytic anaemias. The virus multiplies in the developing red cells of the bone marrow, destroying them and causing severe anaemia.

Parvovirus B19 has also been reported to cause erythema infectiosum (the fifth disease) a moderately contagious disease of children characterised by erythematous rash first appearing on the cheeks (slapped cheek appearance). Animal parvovirus infections frequently cause severe birth defects but there is no evidence of this in humans. Human infection with the virus is widely prevalent and generally asymptomatic.

RUBELLA

Rubella or German measles is a mild exanthematous fever characterised by transient macular rash and lymphadenopathy. In itself, the disease is trivial but rubella in the pregnant woman may lead to congenital malformations in the baby. The teratogenic property of rubella was discovered by an Australian ophthalmologist Gregg, who in 1941 observed a sudden increase in congenital cataract in infants and related it to maternal rubella. Observations from different countries soon confirmed that maternal rubella induces congenital malformations of different kinds, the commonest being the triad of cataract, deafness and cardiac defects. Though experimental transmission of the disease to human volunteers and monkeys was achieved, progress was hampered as the virus could not be cultivated. Rubella virus was isolated in tissue culture in 1962, independently by two groups of workers (Weller and Neva; Parkman, Buescher and Artenstein).

Properties of the virus: Rubella virus is a pleomorphic, roughly spherical particle, 50–70 nm in diameter, surrounded by an envelope and containing RNA. It agglutinates goose, pigeon and day old chick erythrocytes at 4°C. Structurally and in its haemagglutinating property, rubella virus resembles togaviruses, but they are unrelated serologically. Further the absence of any arthropod vector excludes rubella virus from the arbovirus group. Rubella virus has been classified in the genus *Rubivirus* in the family 'Togaviridae'.

The virus is inactivated by ether, chloroform, formaldehyde, beta propiolactone and desoxycholate. It is destroyed by heating at 56°C, but survives for several years at −60°C.

Cultivation and host range: Virus growth in tissue culture can be detected, depending on the type of cells used, either by interference or by cytopathic effects. In African green monkey or patas monkey kidney, or HeLa cells, cytopathic effects are not observed and virus growth can be identified by interference, using a challenge virus such as echo 11. Focal cytopathic changes are produced in continuous cell lines derived from rabbit (RK 13) or hamster (BHK 21) kidney.

Experimental infection can be produced in human volunteers and monkeys. A suitable experimental model for the teratogenic effects of rubella is the pregnant rabbit, in which the virus infects the fetus transplacentally, leading to congenital malformations.

Clinical features: Rubella is a relatively mild disease. After an incubation period of 2–3 weeks generalised rash occurs, appearing first on the face and then spreading to the neck, trunk and extremities sparing the palms and soles. The rash is generally discrete and rarely coalesces and ordinarily disappears by the third day. There is nontender enlargement of posterior cervical glands — auricular, suboccipital and cervical. Koplik's spots are absent unlike in measles. Catarrhal symptoms and mild fever may precede the rash. The disease occurs principally in children but may affect all ages. The major complications are thrombocytopaenic purpura, meningoencephalitis, arthralgia and arthritis, the latter two are commoner in women and with increasing age. Based on the results of *in vitro* studies, rubella infection is presumed to result in chromosomal breakages and inhibition of mitoses in infected embryonic cells.

Infection is acquired by inhalation. Virus can be recovered from the throat for about seven days before the rash. It has been recorded as early as thirteen days before the rash and is constantly present for five days after the rash. Viraemia has been demonstrated as early as the seventh day before the rash and ceases shortly after the appearance of the rash. Virus can also be demonstrated in the faeces and the urine. Patients with subclinical infection are infectious and also develop viraemia. Infection of the fetus is by the bloodstream.

Fetal damage caused by maternal rubella is related to the stage of pregnancy. If rubella occurs in very early pregnancy, the fetus may die. Congenital malformations are commonest during the first trimester. Later, the damage caused may be more subtle, in the form of communication defects or developmental retardation and may not be apparent till the child grows older. The commonest malformations caused by rubella are cardiac defects, cataract and deafness. Multiple defects are common. Several other features have been recognised in babies with congenital rubella. These include hepatosplenomegaly, thrombocytopaenic purpura, myocarditis and bone lesions, and constitute the 'expanded rubella syndrome'.

Rubella virus is present in all excretions of congenitally infected infants. About a third of them continue to shed virus for six months, and a few for a year. Virus may persist in tissues such as cataractous lenses for several years. Infected babies constitute an important source of infection to the staff in nurseries.

Laboratory diagnosis: Routine diagnosis of rubella is not called for, but laboratory confirmation becomes important when rubella is suspected in pregnant women. Diagnosis can be established by virus isolation or serology. Virus may be isolated from the blood during the early stage or more successfully from throat swabs. Rabbit kidney or vero cell cultures are inoculated and virus growth detected by interference or cytopathic changes. The virus grows better if cultures are incubated at a lower temperature, 33°–35°C. Serological diagnosis depends upon testing paired sera by neutralisation, haemagglutination inhibition, complement fixation or immunofluorescence tests. ELISA and radial immunohaemolysis are useful as screening tests.

In congenital rubella, the virus may be isolated from a variety of sources such as urine, throat swabs, leucocytes, bone marrow or cerebrospinal fluid. Serological diagnosis is made by demonstrating IgM antibodies. These indicate immune response in the fetus as against IgG, antibodies which may be passively transferred from the mother. Demonstration of IgM antibody is important also in pregnant women who develop exanthematous disease suspected to be rubella. Diagnosis of rubella in early pregnancy is an indication for therapeutic abortion.

Prophylaxis: Rubella infection confers lasting immunity as there is only one antigenic type of the virus. Reinfections have, however, been reported. The disease being so mild, prophylaxis is directed only towards its teratogenic hazard and so relevant only in women of child bearing age. An obvious method of protection is to acquire the infection before puberty. This was achieved by 'rubella parties', formerly practised in Australia, where adolescent girls voluntarily exposed themselves to known rubella cases.

Live attenuated vaccines have been developed by serial passage of the virus in tissue culture. The vaccines in use now are RA 27/3 produced in human diploid cell culture and the Cendehill vaccine attenuated in rabbit kidney culture. They are given subcutaneously. RA 27/3 vaccine induces antibody response when administered intranasally also, but it has not been licensed for use by this route. This vaccine may be given along with measles and mumps vaccines (MMR vaccine). The vaccine is generally well tolerated, though minor reactions like lymphadenopathy, rash and arthralgia may sometime occur. It should not be given to immunodeficient subjects. Pregnancy is an absolute contraindication and should be avoided for three months after vaccination. Passive immunisation is of limited value.

Epidemiology: Rubella is worldwide in distribution. Serological surveys in different countries have shown that 80–90 per cent are immune by the age of 15 years. About 10–20 per cent of mothers are nonimmune and therefore vulnerable.

SLOW VIRUS DISEASES

The term 'slow virus diseases' is applied to a group of infections in animals and man, characterised by a very long incubation period and a slow but relentless course, terminating fatally. The concept of 'slow infection' was originally proposed by Sigurdsson (1954), a veterinary pathologist for slowly progressing infections of sheep, such as scrapie, visna and maedi. The recognition in recent years, that some chronic degenerative neurological diseases of man may have a similar pathogenesis, has led to considerable interest in this concept.

Slow virus infections have the following characters: 1) Incubation periods ranging from months to years, 2) course of illness lasting for months or years, with remissions and exacerbations, 3) predilection for involvement of the central nervous system, 4) absence of immune response or an immune response that does not arrest the disease, but may actually contribute to pathogenesis. 5) a genetic predisposition, and 6) invariable fatal termination.

Slow virus diseases may be classified into three groups:

Group A consisting of slowly progressive infections of sheep, caused by serologically related nononcogenic retroviruses called *lentiviruses* (from (L) *lentus* meaning slow), *Group B* comprising four obscure infections of the CNS, scrapie, mink encephalopathy Kuru and Creutzfeldt-Jakob disease, collectively known as the 'subacute spongiform viral encephalopathies' and *Group C* consisting of two unrelated CNS diseases of man, subacute sclerosing panencephalitis and progressive multifocal leucoencephalopathy.

Group A: Visna, a demyelinating disease of sheep was originally recognised in Iceland in 1935 where it was eradicated in 1951 by slaughter of all affected animals. The disease has an incubation period of about two years. It has an insidious onset with pareses, progressing to total paralysis and death. The virus can be grown in sheep choroid plexus tissue cultures, from CSF, blood and saliva of affected animals. High levels of neutralising antibody can be detected in circulation, but they do not protect the host. It has been suggested that the CNS lesions may represent an immune disease, due to an antigen-antibody reaction on the surface of infected glial cells.

Maedi (progressive pneumonia) is a slowly progressive fatal haemorrhagic pneumonia of sheep, with an incubation period of 2–3 years.

1. South American haemorrhagic fevers: Two related viruses, the Junin and Machupo viruses cause the Argentinian and Bolivian haemorrhagic fevers, respectively. They belong to the Tacaribe group of arenaviruses. Rodents act as reservoirs and transmission is believed to occur through rodent excreta.

2. Lassa fever: This is the most highly publicised of viral haemorrhagic fevers and is caused by another arenavirus. It was first noticed in 1969 in an American Mission station in Lassa, Nigeria. Many outbreaks have subsequently occurred in widely separated foci in West Africa. The case fatality rate has been 35–70 per cent in hospitalised patients. Serological surveys indicate that Lassa virus is endemic widely in West Africa, causing asymptomatic infection in the native population. Natural reservoir is the multimammate rats. Rodent excreta probably act as the source of infection. The incubation period is 3–16 days. The virus is present in the throat, urine and blood of patients. Person to person transmission may occur by droplet infection. Nosocomial infection has occurred frequently. Ribavirin has proved useful in treatment.

3. Marburg disease and Ebola fever: Marburg disease is a haemorrhagic fever that occurred simultaneously in laboratory workers in Marburg, Frankfurt (Germany) and Belgrade (Yugoslavia) in 1967. The infection arose from tissues of African green monkeys to which the laboratory workers had been exposed. The monkeys had been recently imported from Uganda. Person to person transmission occurred. The primary cases had a fatility rate of 30 per cent, but the secondary cases were nonfatal.

Marburg virus was isolated in guinea pigs and tissue culture from the blood and tissues of these patients. The virus appears to persist in the body and has been isolated after 80 days of onset of illness from semen and the anterior chamber of the eye. A case of sexual transmission has been recorded.

No further Marburg virus infection was seen except for three cases identified in South Africa in 1975 and in two in Kenya in 1980.

In 1976, several cases of haemorrhagic fever, similar to Marburg disease, occurred in the equatorial provinces of Sudan and Zaire, with high fatality. The causative virus was morphologically identical to Marburg virus, but antigenically distinct. It has been called Ebola virus. In 1979 Ebola reemerged in the Sudan, with serial man to man spread.

Marburg and Ebola viruses are RNA viruses resembling rhabdoviruses structurally, but more pleomorphic with long tubular forms. They have been provisionally assigned to a new family called *Filoviridae*. The natural reservoir of Marburg and Ebola viruses is not known, but is presumably some African rodent.

4. Haemorrhagic fever with renal syndrome (HFRS): This disease, also known as epidemic haemorrhagic nephrosonephritis and Far Eastern or Korean haemorrhagic fever, has been known to Russian and Japanese workers for several decades. It attracted wide attention during the Korean war following infections among the United Nations troops. After an incubation period of two weeks, the illness begins abruptly with fever and proteinuria, followed by haemorrhagic manifestations. The virus has been isolated from blood and urine of patients and from striped field mice which constitute the reservoir hosts. The virus, named *Hantaan virus* is a medium sized, enveloped, acid sensitive, RNA virus belonging to the family '*Bunyaviridae*'. The infection is believed to spread to man from rodent excreta. Person to person transmission does not seem to occur.

Infection with Hantaan virus and antigenically related viruses appear to be common in many areas of Asia, Europe and America, producing haemorrhagic fever only very rarely, but more commonly causing renal damage. Because rodents act as reservoirs for these viruses, the name 'muroid virus nephropathies' or 'rodent-borne nephropathy' has been suggested for this zoonosis.

CORONAVIRUSES

A group of spherical or pleomorphic enveloped RNA viruses, carrying on their surface, petal or club shaped peplomers has been classified as coronaviruses. The name refers to the fringe of surface projections surrounding the virus, resembling the solar corona. The group originally contained veterinary pathogens such as avian infectious bronchitis virus, mouse hepatitis virus and transmissible gastroenteritis virus of swine. Human coronaviruses were first isolated from cases of common cold by inoculating organ cultures of human embryonic trachea with nasopharyngeal washings. Inhibition of ciliary motility indicates virus growth. Though many strains grow only on organ cultures, some grow on monolayers of diploid human embryonic fibroblasts, with minimal cytopathic effects. Many serotypes of human coronaviruses have been recognised. Inoculation in human volunteers induces common cold after an incubation period of 2–5 days. The resulting immunity is poor and reinfections can occur even with the same serotype. Coronaviruses appear to be the second most common cause of common cold, particularly in winter, next only to rhinoviruses.

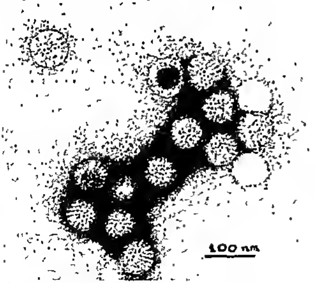

Fig 60.1 Rotavirus in stool x 200,000 (Courtesy: National Institute of Cholera and Enteric Disease, Calcutta, 700 010)

Coronaviruses are established causes of diarrhoea in calves, piglets and dogs. Coronaviruses have been observed in human faeces also, sometime in large numbers, but their significance is not known.

REOVIRIDAE

The family Reoviridae derives its name from the prototype virus which was known as the Respiratory Enteric Orphan virus, because it could be isolated frequently from the respiratory and enteric tracts, and it was considered an 'orphan' as its pathogenicity for man was not proven. Members of this family are double shelled icosahedral viruses, 55–75 nm in diameter. The genome consists of double stranded RNA in 10–12 pieces, a feature unique among animal viruses. They are nonenveloped and resistant to lipid solvents. The family contains three genera — *Reovirus, Orbivirus* and *Rotavirus.*

Reovirus: The genus *Reovirus* contains three mammalian serotypes (Reovirus types 1,2 and 3) which possess a common complement fixing antigen, but can be differentiated by neutralisation tests. Reoviruses agglutinate human erythrocytes. Haemagglutination is type specific and is neutralised by the specific antibody. Reoviruses have not been proved to cause any human disease.

Orbivirus These have a double shell in which the outer layer is fuzzy and indistinct. The inner layer has 32 ring shaped capsomers. (The name Orbivirus is derived from *Orbi*, in Latin, meaning ring). Orbiviruses multiply in insects and vertebrates, thus qualifying as arboviruses. They are responsible for veterinary disease such as African horse sickness and blue tongue. The only known orbivirus infection of man is Colorado tick fever, a minor febrile illness.

Rotaviruses: These double walled viruses present a characteristic appearance under the electron microscope, resembling little wheels with

short spokes radiating from a wide hub to a clearly defined outer rim. (The name is derived from *Rota*, in Latin, meaning wheel.) Both 'complete' and 'incomplete' particles are seen. The complete virus measures about 65–70 nm in diameter and has a smooth surface. The 'incomplete' virus is smaller, about 60 nm, with a rough surface and is believed to be rotavirus that has lost the outer shell.

Bishop and colleagues (1973) in Melbourne reported rotaviruses in ultrathin sections of duodenal mucosal biopsies from acute infantile gastroenteritis, when examined under the electron microscope. Soon after, Flewett and colleagues in Birmingham also observed similar viruses in faecal extracts from infantile gastroenteritis cases, by electron microscopy, using negative staining techniques. These observations have been confirmed and rotaviruses are now recognised as the most common cause of diarrhoeal disease in infants and children.

The methods used for diagnosis were originally electron microscopy and immunoelectron microscopy. These are expensive and complicated procedures. Concentrations of more than 10^6 particles per ml are necessary for the virus to be seen in faeces by electron microscopy. Serological techniques for demonstration of the virus in stools are simpler and as sensitive. Complement fixation, counterimmunoeletrophoresis, enzyme linked immunosorbent assay and radioimmunoassay are used for this purpose. IgM and IgG antibodies can be demonstrated in the blood of infected children. Rotaviruses share a common group antigen situated in the inner capsid layer. Human rotaviruses have been classified into two *subgroups* (I and II) by ELISA, CF or immune adherence agglutination, and into four serotypes (1,2,3,4) by neutralisation tests. By polyacrylamide gel electrophoresis, rotavirus strains can be classified into several electrophore types, based on the patterns of migration of the viral RNA.

Rotaviruses are now known to be a class of viruses causing diarrhoea in the young of many animals and some birds. The human rotavirus is related to the viruses of epidemic diarrhoea of infant mice (EDIM), Nebraska calf diarrhoea and the simian virus SA 11. All rotaviruses share common antigens. Though the viruses are in general species specific, interspecies infection can be induced experimentally. Human rotavirus infection has been transferred to piglets, calves and monkeys. It is not known whether human infection can be caused by animal rotaviruses.

Human rotavirus does not grow readily in cell cultures, but some strains have been adapted for serial growth in tissue cultures. Rotavirus growth is facilitated by trypsin treatment and rolling of tissue cultures. As calf and simian viruses grow readily in cell cultures, they have been used as antigens for serological studies.

Rotaviruses are the commonest cause of diarrhoea in infants and children the world over and account for about half the cases of children hospitalised for diarrhoea. It occurs throughout the year, but predominates in winter months, when the virus may be detected in some 75 per cent of the patients. It sometimes produces large epidemics of diarrhoea in winter. Rotavirus diarrhoea is usually seen in children below the age of five years, but is most frequent between six and 24 months of age. Infection is not infrequent in neonates, but they seldom develop diarrhoea, perhaps because of maternal passive immunity. By the age of five years, most children have had clinical or subclinical infection, so that rotavirus diarrhoea is very uncommon in older children and adults.

Infection is by the faecal-oral route. The incubation period is 2–3 days. Vomiting and diarrhoea occur with little or no fever. Stools are usually greenish yellow or white, with no blood or mucus. The disease is self-limited and recovery occurs within 5–10 days. Mortality is low. Rehydration is all the treatment needed.

OTHER VIRUSES CAUSING DIARRHOEA

As bacterial and parasitic agents accounted only for about a quarter of all diarrhoea cases, viruses had for long been suspected as common causes of

diarrhoeal disease. Early attempts to identify viral agents in diarrhoea, using tissue culture methods were unrewarding. A number of enteroviruses and adenoviruses were incriminated in diarrhoea but the evidence against them remained inconclusive. Besides rotaviruses, the following viruses are known or suspected to cause diarrhoeal disease.

Norwalk virus: A 27 nm virus was shown to be responsible for an epidemic gastroenteritis affecting school children and teachers in Norwalk, Ohio, in 1968. The virus induces diarrhoea in human volunteers. Serological surveys have shown that infection with Norwalk virus is widespread in many countries. Extensive epidemics of Norwalk virus diarrhoea associated with consumption of raw oysters have been reported from Australia and America. Norwalk virus appears to be an important cause of diarrhoea in adults and children.

The virus can be demonstrated in faeces by electron microscopy. Antibody to the virus can be detected by immune electron microscopy and radioimmunoassay. It has not yet been propagated in cell cultures. Little is known about the properties of the virus. It has been included in the family *Caliciviridae* consisting of small round RNA viruses, 22-30 nm in size. The name calicivirus is derived from the presence of 32 cup shaped depressions on the virus surface (from *calyx*, meaning cup).

Adenovirus: Several outbreaks of diarrhoea in children have been associated with the presence of large numbers of adenoviruses in faeces. These adenoviruses can be grown only with difficulty in tissue culture. They have been designated types 40 and 41. Adenovirus associated diarrhoea has been seen more often in summer months.

Astrovirus: These star shaped, 28 nm isometric particles have been found associated with some epidemics of diarrhoea in children. Similar viruses have also been identified in lamb and calf diarrhoea.

Coronavirus: These are well established causes of acute diarrhoea in calves, piglets and dogs. They have been observed in human faeces also but their relation to diarrhoea is uncertain.

Caliciviruses and a heterogeneous group of 22-30 nm viruses ('small round viruses') have been reported as possible causative agents of diarrhoea, more in association with localised outbreaks than with sporadic disease.

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61 Oncogenic Viruses

The association of viruses with malignancy dates from the discovery by Ellerman and Bang (1908) of the viral aetiology of fowl leukaemia. Rous (1911) showed that a solid malignant tumour, fowl sarcoma, was caused by a virus. Viruses caussing tumours in animals were first demonstrated by Shope, who isolated the rabbit fibroma virus in 1932 and papilloma virus in 1933. Though these are benign tumours, papillomas may turn malignant. Bittner (1936) proposed that breast cancer in mice could be caused by a virus transmitted from mother to offspring through breast milk. During the 1950s many viruses were identified which induced leukaemia in rodents. Considerable interest was aroused by the discovery by Stewart and Eddy (1957) of the polyoma virus which could produce a wide variety of neoplasms when injected into newborn rodents. Injection of certain types of human adenovirus into newborn hamsters was shown by Trentin (1962) to cause sarcomas. Burkitt (1963) identified a peculiar geographical distribution of lymphomas in African children and suspected that it may be caused by a virus transmitted by an insect. The Epstein-Barr virus isolated from Burkitt's lymphoma has been proposed as the aetiological agent. Many viruses have been isolated from human cancer or demonstrated electron microscopically in affected cells and tissues, but most of them were merely 'passenger' viruses present in the lesions and not the causative agents.

Viruses that produce tumours in their natural hosts or in experimental animals, or induce malignant transformation of cells on culture, are known as oncogenic viruses. Transformation represents the various changes that accompany the conversion of a normal cell into the malignant cell (Table 61.1). Transformation is recognised primarily by a change in morphology of cultured cells.

Transformed cells are altered in shape and lose the property of 'contact inhibition' so that, instead of growing as monolayer, they grow piled up, one over another, forming 'microtumours'. Foci of transformation can be easily made out and are used in the assay of oncogenic viruses, such as Rous sarcoma virus.

About a quarter of the 600 or so animal viruses possess oncogenic potential (Table 61.2). The viruses associated with cancers in humans are shown in Table 61.3. Both RNA and DNA viruses are oncogenic. While all oncogenic RNA viruses (formerly called oncornaviruses) belong to a single family (retrovirus), oncogenic viruses occur among many genera of DNA viruses. Retroviruses are responsible for naturally occurring leukaemia and sarcoma in several species of animals. Among DNA viruses, some herpes viruses cause malignant tumours in their natural hosts.

Oncogenic RNA viruses

Retrovirus: All oncogenic RNA viruses belong to the family Retroviridae, the name being derived from the presence in the viruses, of the unusual enzyme, *reverse transcriptase* which is RNA directed DNA polymerase. Reverse transcriptase prepares a DNA copy of the RNA genome of the virus. The DNA copy becomes

TABLE 61.1
Properties of cells transformed by viruses

I. Altered cell morphology:
Fibroblasts become shorter, parallel orientation is lost, *chromosomal aberrations appear*.

II. Altered cell metabolism:
Increased growth rate, increased production of organic acids and acid mucopolysaccharides.

III. Altered growth characteristics:
Loss of contact inhibition, formation of *heaped-up growth* (microtumours), capacity to divide indefinitely in serial culture, capacity to grow in suspension or in semisolid agar.

IV. Antigenic alterations:
Appearance of new virus specified antigens (T antigen — TSTA)
Loss of surface antigens, cells become agglutinable by lectins

V. Capacity to induce tumours in susceptible animals

TABLE 61.2
List of oncogenic viruses

A. RNA VIRUSES:
- I. Retroviruses:
  1. Avian leukosis viruses
  2. Murine leukosis viruses
  3. Murine mammary tumour virus
  4. Leukosis-sarcorma viruses of various animals
  5. Human T cell leukaemia virus

B. DNA VIRUSES:
- I. Papovavirus:
  1. Papillomaviruses of man, rabbits and other animals
  2. Polyomavirus
  3. *Simian virus 40*
  4. BK and JC viruses
- II. Poxvirus:
  1. Molluscum contagiosum
  2. Yaba virus
  3. *Shope fibroma*
- III Adenovirus
  Many human and nonhuman types
- IV. Herpesvirus
  1. Marek's disease virus
  2. Lucke's frog tumour virus
  3. Herpes virus saimiri
  4. Epstein-Barr virus
  5. Herpes simplex virus types 1 and 2
  6. Cytomegalovirus
- V. Hepatitis B virus

TABLE 61 3

Viruses associated with human cancer

Virus	Human cancer
Herpesviridae. EB virus	Nasopharyngeal carcinoma.
	Burkitt's lymphoma. B cell lymphoma
HSV2	Cervical carcinoma.
Papovaviridae	Urogenital tumours.
Papilloma virus	Penile, vulval, cervical cancers.
Hepadnaviridae HBV	Primary hepatocellular carcinoma.
Retroviridae HTL virus	Adult T cell leukaemia.

integrated into the DNA of the infected host cell and it is from this DNA copy (provirus) that all proteins of the virus are translated. The family Retroviridae is divided into the subfamilies: *Oncovirinae* containing all oncornaviruses; *Lentivirinae*, containing some nononcogenic viruses causing slow virus infections; and *spumivirinae* which contain the nononcogenic 'foamy viruses' causing asymptomatic infection in several species of animals.

Retroviruses are widespread in nature and cause leukosis or sarcoma in their hosts. The viruses are usually transmitted vertically from parent to progeny (congenitally), and less often horizontally (postnatally). Oncogenic retroviruses are classified into five groups:

1. The avian leukosis complex: A group of antigenically related viruses which induce avian leukosis (lymphomatosis, myeloblastosis and erythroblastosis viruses) or sarcoma in fowls (Rous sarcoma virus RSV).

2. Murine leukosis viruses: This group consists of several strains of murine leukaemia and sarcoma viruses, named after the investigators who first described them, e g., Gross, Friend, Moloney, Rauscher.

3. Mammary tumour virus of mice: This virus occurs in certain strains of mice having a high natural incidence of breast cancer. It used to be known as the 'milk factor' or 'Bittner virus'. It multiplies in the mammary gland and is transmitted from mother to offspring through breast milk. Mice can be infected by oral, subcutaneous or intraperitoneal routes. Mammary cancer occurs only in susceptible strains of mice, after a latent period of 6–12 months.

4 Leukosis-sarcoma viruses of other animals: A number of viruses have been isolated from leukosis and sarcomas in various species of animals — cat, hamster, rat, guinea pig and monkey. Many of them have not been fully characterised.

5. Human T cell leukaemia virus (HTLV): Retroviruses named human T cell leukaemia viruses were isolated in 1981 from cell cultures from adult patients with cutaneous T cell lymphoma (mycosis fungoides) and leukaemia (Sezary syndrome) in the USA. Similar viruses have been isolated from patients with adult T cell leukaemia in Japan and the Caribbean Antibodies to HTLV are found in many normal persons in endemic areas (particularly in the South Western island of Kyushu in Japan), but not in other areas. Two serotypes of HTLV have been associated with leukaemia/lymphoma while HTLV type III (now called HIV) causes acquired immunodeficiency syndrome (AIDS) where the virus is believed to infect and selectively destroy the helper T cells.

Morphology: Retroviruses are enveloped viruses that develop by budding through the host cell membrane. They are approximately 100 nm in size. They contain single stranded RNA with a sedimentation coefficient of 70S. Retroviruses are classified into four types based on their mor-

phology. Type A particles occur only intracellularly and consist of a ring shaped nucleoid surrounded by a membrane. They may represent noninfectious precursors of extracellular B type particles or may be distinct entities themselves. B type particles are larger (100–130 nm) with an eccentric nucleoid and carry spikes on the surface membrane. Type C particles have a centrally situated nucleoid and a smooth surface membrane. The D type particles have not been adequatley characterised. They occur as intracellular and extracellular forms, mature as well as immature. The mature particles contain an eccentric nucleoid and carry short surface spikes. The mouse mammary tumour virus is a B type particle. The Mason-Pfizer monkey mammary carcinomavirus is a D type particle. All the other retroviruses listed above are C type particles.

Retroviruses are readily inactivated by heating (56°C for 30 mins), mild acid treatment and by formalin. They are ether sensitive. They are stable at -30°.

Two types of antigens are present in retroviruses; type specific antigens associated with the viral envelope and group specific antigens associated with the nucleoprotein.

Oncogenic DNA viruses

Papovaviruses: Papilloma viruses cause benign tumours in their natural hosts, but some of them (e.g., condyloma acuminatum in man, rabbit papilloma) may turn malignant. There is evidence of association between human papilloma virus (HPV) infection and cancer of cervix uteri, particularly HPV types 16 and 18. In general, infectious virus particles cannot be demonstrated in tumours induced by DNA viruses, but papilloma in the wild cotton-tail rabbit is an exception. Rabbit papilloma virus, or DNA extracted from it, can produce papilloma in rabbits following subcutaneous injection.

Polyoma virus causes natural latent infection in laboratory and domestic mice. But, when injected into infant mice or other rodents, it induces a wide variety of histologically diverse tumours. The virus can be cultivated in mouse embryo fibroblasts or baby hamster kidney cells, in which it induces transformation. Polyoma virus produces a haemagglutinin.

Simian virus 40 (SV 40) was discovered in apparently normal monkey kidney cultures used for production of polio vaccine. It causes an inapparent infection in rhesus and cynomolgus monkeys and does not cause cytopathic effects in cell cultures from such monkeys. But, when fluid from such cultures is inoculated into renal cell cultures derived from African green monkeys, cytopathic change with prominent cytoplasmic vacuolation results. Injection into newborn hamsters produces tumours. Transformation is induced in cultured cells from several species, including human cells. Millions of doses of polio vaccine prepared in monkey kidney cultures that may have harboured SV 40 virus had been used before the virus was discovered. These individuals have been followed up for over 25 years and no SV 40 related tumours have been reported. There was

Fig 61.1 Mouse mammary cancer, section stained with uranyl acetate and lead nitrate, showing mature budding B type virus (arrow) and immature intracellular A type virus (triple arrow) x 150,000 (Courtesy: Ultrastructure division Cancer Research Institute, Bombay)

considerable apprehension, when the oncogenic effect of SV 40 was discovered. But there is no evidence that injection of vaccine containing SV 40 has induced cancer in man.

Poxvirus: Three members of the poxvirus group induce benign tumours, rabbit fibroma, molluscum contagiosum and the Yaba virus. The latter causes naturally occurring benign histiocytomas in monkeys. It is apparently transmitted by insects. Similar tumours can be induced experimentally in many species of primates, including man. The tumours regress spontaneously in a few weeks. Nonprimates are insusceptible.

Adenovirus. Though some types (12,18,21) of human adenovirus may produce sarcomas in newborn rodents after experimental inoculation, they do not appear to have any association with human cancer.

Herpesvirus: Many herpesviruses have been associated with natural cancers in animals and man.

a. Marek's disease: Marek's disease is a highly fatal contagious neurolymphomatosis of chickens. No infectious virus particle can be isolated from the lesions or seen under the electron microscope. But sick birds shed large quantities of virus from their feather follicles. The virus is a typical herpes virus. Marek's disease can be induced in young chicken by injection of the virus. The virus grows well in chick embryo fibroblasts producing cytopathic changes, but no evidence of transformation. Marek's disease can be prevented by a live avirulent vaccine. This is the first instance of a malignant disease being controlled by a viral vaccine.

b. Lucke's tumour of frogs: A herpesvirus is considered to be the aetiological agent of a renal adenocarcinoma in frogs.

c. Herpesvirus saimiri: This virus was isolated from a culture of squirrel monkey kidney cells. It causes fatal lymphoma or reticulum cell sarcoma when injected into owl monkeys or rabbits. *Herpesvirus saimiri* infection has been suggested as a primate model for the study of interactions between EB virus and man.

d. Epstein-Barr virus: A herpesvirus, called the Epstein-Barr virus, is found regularly in cultured lymphocytes from Burkitt's lymphoma patients. In the body the tumour cells contain no virus but cell lines established from them almost always contain 5–20 per cent of cells that produce virus. The virus multiplies only in human lymphoid cell lines. Serological surveys show that infection with the virus is worldwide. Infection is usually asymptomatic. In young adults without preexisting antibodies, EB virus infection induces infectious mononucleosis. Lymphoma is believed to occur when the infection takes place in children whose immune system in compromised, as for instance, by chronic malaria. EB virus associated lymphomas have been reported in transplant recipients. EBV has also been linked to nasopharyngeal carcinoma in the Chinese male population in South East Asia and East Africa.

e. Herpes simplex and cancer cervix: A strong association has been reported between herpes simplex type 2 infection and cancer of the uterine cervix. A higher incidence of antibody to the virus has been demonstrated in women with cervical cancer than in controls. The virus has been isolated more often from the genital tract of cancer patients than from controls. Though circumstantial evidence has been gathered, aetiological relationship has not been proved.

It has also been suggested that herpes simplex type 1 infection may be associated with cancer of the lip.

f. Cytomegalovirus infection has been suggested to be associated with carcinoma of the prostate and Kaposi's sarcoma.

Hepatitis B virus: HBV has been claimed to be directly or indirectly involved in the aetiology of hepatocellular carcinoma. Studies in many countries have demonstrated an excess prevalence of markers of HBV infection in patients with primary hepatocellular carcinoma as compared with matched controls or with the general population.

MECHANISMS OF VIRAL ONCOGENESIS

While it is knnwn that oncogenic viruses are able to cause transformation of cells in culture and induce tumours in animals, under natural or experimental conditions, the exact mechanisms of viral oncogenesis are obscure. Malignancy is a stable heritable change and, as such, should be the result of a modification of the host cell genome.

In the case of oncogenic DNA viruses, the viral DNA (or a portion of it) is integrated with the host cell genome. The viral DNA being incomplete or 'defective', no infectious virus is produced. But under its influence, the host cell undergoes malignant transformation. A virus transformed cancer cell is in many ways analognus to a hacterium lysogenised by a defective phage. In both cases, the cell is not destroyed and no virus is produced. Acquisition of new characters by the transformed cell resembles lysogenic conversion in hacteria.

In the case of oncogenic retroviruses, the viral RNA is first converted into an RNA:DNA hybrid. This is due to the action of the unusual enzyme RNA directed DNA polymerase (reverse transcriptase) which is characteristic of all retroviruses. The RNA:DNA hybrid is in turn converted into the double stranded DNA form by DNA directed DNA polymerase. This douhle stranded DNA version of the retrovirus genome is called *provirus* and is integrated into the host cell genome. The integrated provirus acts as a template for viral RNA synthesis and also induces cell transformation.

The transforming ability of retroviruses is due to specific genes carried by them. Genes that are responsible for the induction of tumours are called *oncogenes* (commonly referred to as cancer genes). Oncogenes are not essential for the replication of retroviruses, and mutants occur that lack oncogenes. Such mutants can replicate normally hut are incapable of transforming cells or inducing tumours. For example, Rous sarcoma virus (RSV) carries the oncogene *src*, which is responsible for its transforming and tumour producing capacity. RSV mutants lacking *src* replicate normally, but are not oncogenic.

The oncogenes present in retroviruses are called *viral oncogenes* (V-onc). Genes with sequences similar to retroviral oncogenes exist in host cells also, not only in tumour cells but also in normal cells of man, animals and birds. These genes are called *cellular oncogenes* (C-onc). Cellular oncogenes are not of viral origin. On the contrary, it has been established that viral oncogenes are in fact of host cell origin. Cellular oncogenes are widespread in vertebrates and are well conserved at constant sites in their genomes. They

TABLE 61.4
Oncogenes * and their chromosomal location in man

Viral oncogene	Origin	Natural tumour	Human gene	Chromosomal location in man
V-*src*	chicken	sarcoma	C-*src*	20
V-*ras*	rat	sarcoma	C-*ras*	11
V-*myc*,	chicken	leukaemia	C-*myc*	8
V-*fes*	cat	sarcoma	C-*fes*	15
V-*sis*	monkey	sarcoma	C-*sis*	22
V-*mos*	mouse	sarcoma	C-*mos*	8

(* Oncogenes are given 3-letter codes from the animal or tumour from which they are derived, preceded by either V-or C-, for viral or cellular genes respectively; src = sarcoma of chicken, ras = rat sarcoma, myc = myelomatosis of chicken, fes = feline sarcoma, sis = simian sarcoma, mos = mouse sarcoma.).

contain introns characteristic of eukaryotic genes. It is believed that retroviruses may have picked up these oncogenes from host cells during infection at some remote time during evolution (Table 61.4).

Cellular oncogenes are believed to have some important controlling function on cell growth and regulation. Some viral oncogenes also code for substances that influence cell growth and regulation. For example, *V-src* oncogene of RSV codes for a phosphoprotein, a phosphokinase which phosphorylates tyrosine. This phosphoprotein appears to be concentrated along the plasma membranes of infected cells and in adhesion plaques which anchor cells to surfaces, indicating that it may influence cell growth and replication.

One of the methods for studying oncogenes is by *transfection*. Certain mouse fibroblast cell lines (e.g., NIH 3T3) can take up foreign DNA, incorporate them into their genome and express them. This method of gene transfer is called transfection. By this technique, DNA extracted from human tumour cells has been shown to transform 3T3 cells, and such transforming genes have been shown to be identical with cellular oncogenes

Not all oncogenic retroviruses contain oncogenes. Some such as avian leukosis virus initiate oncogenesis by activating host cell oncogenes (oncogenesis by promoter insertion).

Cancer is not one disease and not all cancers may have the same cause. It is possible that the various theories of the origin of cancer—viruses, chemicals, environmental factors, heredity, immunological factors, etc. — may all be true in parts. They are not mutually exclusive. As a unified theory of oncogenesis, it has been suggested that cancer results when cellular oncogenes overact following infection with oncogenic viruses, or when their expression is modified by the effects of other carcinogens.

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62 Human Immunodeficiency Virus: AIDS

The emergence and pandemic spread of the *acquired immunodeficiency syndrome* (AIDS) constitute the greatest challenge to public health in modern times. After the sudden appearance of syphilis in Europe five hundred years ago, rarely has any new disease had as great an impact on medicine, science and society and caused as much panic among the public and governments as has AIDS. The full consequence of this phenomenon may not be evident for several years because of the silent spread and slow evolution of this infection, but it is truly global in its magnitude and is unlikely to spare any continent, nation, race or political system.

The first indication of this new syndrome began in the summer of 1981, when reports came from New York and California (USA) of a sudden increase in the incidence of two very rare diseases — Kaposi's sarcoma and *Pneumocystis carinii* pneumonia in young adults who were homosexuals or addicted to heroin or other injected narcotics. They appeared to have lost their immune competence, rendering them vulnerable to overwhelming and fatal infections with relatively avirulent microorganisms, as well as to lymphoid and other malignancies. This condition was given the name '*Acquired Immune Deficiency Syndrome*' (AIDS).

Subsequently the syndrome was also found in Haitian immigrants on the east coast of USA and in some haemophiliacs who had received Factor VIII injections. Cases were also observed in some recipients of blood transfusion, in sexual partners of patients and in babies born to infected women. AIDS was therefore recognised to be transmitted by sexual contact or by transfer of blood or other body fluids. With the rapid increase in the numbers of patients detected in the USA and reports of similar cases from other countries, alarm about AIDS mounted and discovery of its causative agent assumed the highest priority.

Isolation of the aetiological agent was first reported in 1983 by Luc Montagnier and colleagues from the Pasteur Institute, Paris. They isolated a retrovirus from a West African patient with persistent generalised lymphadenopathy, which is a manifestation of AIDS, and called it *Lymphadenopathy Associated Virus* (LAV). It produced lytic infection in fresh peripheral blood lymphocytes, but could not be established in permanent cell lines. In 1984, Robert Gallo and colleagues from the National Institute of Health, USA reported isolation of a retrovirus from AIDS patients and called it '*Human T cell Lymphotropic Virus - III* or HTLV-III. Retroviruses HLTV-I and II had already been described earlier in association with human T cell leukaemia. HTLV-III could be grown in continuous culture in T cell leukaemia cell line, yielding sufficient antigen for serological tests. Other similar isolates were reported from AIDS cases under different names such as *AIDS-Related Virus (ARV)* etc. Serological analysis and molecular cloning established the common origin of these viruses inspite of varying degrees of antigenic differences between isolates which had been given different names earlier. To resolve this nomenclatural confusion, the International Committee on Virus Nomenclature in 1986 decided on the generic

1986 HIV

name '*Human Immunodeficiency Virus* (HIV)' for these viruses.

In 1984, the first serological tests became available for detection of anti-HIV antibodies. This made possible a more realistic estimation of the extent of HIV infection. Till then, the infection could be recognised only when patients developed the characteristic, clinical features such as opportunistic infections or malignancies. These end stage cases represented only the tip of the iceberg. Serological screening of high risk groups, blood donors and others revealed a very large and expanding reservoir of HIV in patients and carriers in different parts of the world. It has been stated that somewhere in the world, one person gets infected with HIV every minute. By the end of 1988 over 10 million persons are believed to have been infected by HIV. Some 150,000 of them, in 142 countries representing all continents have developed clinical AIDS. These figures are bound to be underestimations, but they indicate the dimension of the problem which is bound to enlarge with each succeeding year.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

HIV, the aetiological agent of AIDS, belongs to the Lentivirus subgroup of the family Retroviridae. The Lentivirus subgroup (from L. *lentus* = slow) includes the causative agents of the slow virus diseases, visna/maedi in sheep, arthritis/encephalitis in goats and infectious anaemia in horses. Besides HIV, the related animal immunodeficiency viruses also are assigned to this group (Table 62.1)

Structure

HIV is an enveloped virus, 90–120 nm in diameter (Fig. 62.1). It has a nucleoprotein core containing single stranded RNA genome and proteins. In as-

TABLE 62.1
Taxonomy of HIV

	Retroviruses	– RNA viruses with reverse transcriptase enzyme. Classified into 3 subgroups.
I	Spumaviruses	– 'Foamy viruses' found in primary cell cultures. Not associated with disease.
II	Oncoviruses	– RNA tumour viruses. Classified by electron microscopic morphology into A-type (immature), B-type (mammary tumour viruses), C-type (leukaemia viruses including HTLV-I) and D-type (Mason-Pfizer monkey virus) form
III	Lentiviruses	A. Causing degenerative neural disease in animals 1 Visna/maedi in sheep 2 Caprine arthritis/encephalitis in goats. 3 Bovine lentivirus. B Causing immunodeficiency 1 Human Immunodeficiency Virus HIV type 1, type 2. 2. Simian Immunodeficiency Virus (SIV) causing simian AIDS 3. Feline T lymphotropic Virus (FTLV) causing feline AIDS

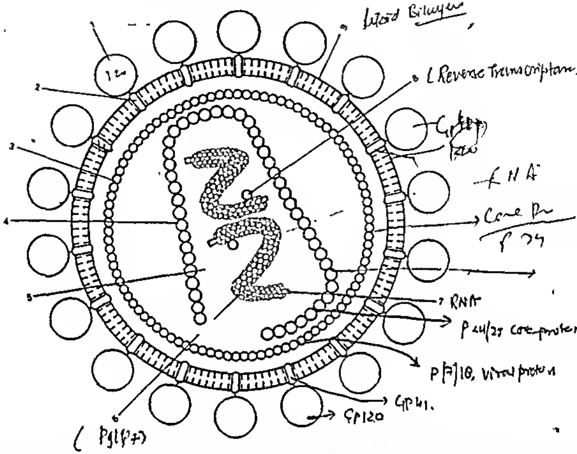

Fig 62.1 Structure of HIV (a diagrammatic representation) A cross-section. 1. Knob of protein gp 120 anchored to another protein gp 41 (marked 2 in the figure). 3. P. 17/18 (viral protein) 4. P 24/25 core protein. 5. Virus core. 6. P 9/P 7 (viral protein) 7. RNA 8. Reverse transcriptase 9. Lipid bilayer.

sociation with viral RNA is the reverse transcriptase enzyme, which is a characteristic feature of retroviruses. When the virus infects a cell, the viral RNA is transcribed by the enzyme, first into single stranded DNA and then to double stranded DNA (provirus) which is *integrated into the host cell chromosome*. The provirus can remain latent for long periods, though it influences host cell functions. At times, in response to viral promoters, the provirus initiates viral replication by directing synthesis of viral RNA and other components.

The virus core is surrounded by a nucleocapsid shell composed of protein. During viral replication when the naked virus buds out through the host cell surface membrane, it acquires a lipoprotein envelope, which consists of lipid derived form the host cell membrane and glycoproteins which are virus-coded. The major virus-coded envelope proteins are the projecting spikes on the surface and the anchoring transmembrane pedicles. The spikes, constitute the major surface component of the virus which binds to the CD4 receptors on susceptible host cells. A

Viral genes and antigens

The genome of HIV contains the three structural genes (*gag*, *pol* and *env*) characteristic of all retroviruses, as well as other non-structural and regulatory genes specific for the virus (Fig. 62.2). The products of these genes, both structural and

non-structural, act as antigens (Table 62.2). Sera of infected persons contain antibodies to them. Detection of these antigens and antibodies is of great value in the diagnosis and prognosis of HIV infections.

A. Genes coding for structural proteins

1. The *gag* gene determines the core and shell of the virus. It is expressed as a precursor protein, p55. (The proteins and glycoproteins are indicated by their mass expressed in kilodaltons.) This precursor protein is cleaved into three proteins, p15, p18 and p24 which make up the viral core and shell. The major core antigen is p24 which can be detected in serum during the early stages of HIV infection before antibodies appear. Late in the course of infection, disappearance of free anti-p24 antibody from circulation points to exacerbation of the illness and is an indication for active antiviral treatment.
2. The *env* determines the synthesis of envelope glycoprotein gp160, which is cleaved into the two envelope components — gp 120 which forms the surface spikes and gp 41 which is the transmembrane anchoring protein. The spike glycoprotein gp120 is the major envelope antigen, and antibodies to gp120 are the first to appear after infection.
3. The *pol* gene codes for the polymerase reverse transcriptase and other viral enzymes. It is expressed as a precursor protein (p100) which is cleaved into proteins p31, p51 and p64.

Non-structural and regulatory genes

1. The *tat* gene, a transactivating factor enhancing viral protein synthesis.
2. The *art* (anti-repressor transactivating) gene promoting viral mRNA translation.
3. Two LTR (*long terminal repeat*) genes that contain sequences giving promotor, enhancer and integration signals.
4. The 3' *orf* (open reading frame) gene exerting a regulatory effect reducing viral replication.
5. The *sor* (short open reading frame) gene encoding a poorly immunogenic protein found in the cytoplasm of infected cells.

Antigenic variation

HIV undergoes frequent antigenic variations involving both core and envelope antigens. Minor antigenic differences are common between isolates from different patients, as well as from the same patient.

Two distinct antigenic types of HIV have been identified. HIV-1 represents the original isolate (LAV/HTLV-III) and related isolates from America, Europe and other Western countries. HIV-2 represents isolates first obtained in 1985

TABLE 62.2
Major antigens of HIV

A.	Envelope antigens:
	1. Spike antigen - gp 120 (Principal envelope antigen) gp 160
	2. Transmembrane pedicle protein - gp41.
B.	Shell antigen
	1. Nucleocapsid protein - p18
C.	Core antigens:
	1. Principal core antigen - p24
	2. Other core antigens - P 15, p55
D.	Polymerase antigens - p31, p51, p64 p 100

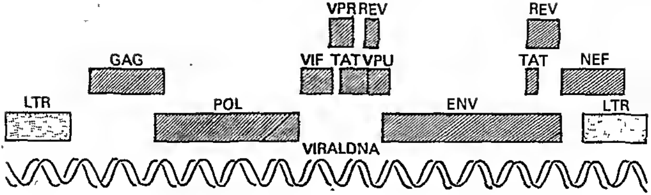

Fig. 62 2 Genetic structure of HIV. The nine genes identified so far which are arranged along the Viral DNA (individual genes shown by striped rectangles). They are flanked by long terminal repeats (LTR's). LTR's initiate expression of other viral genes but do not code for any protein (LTR shown as stippled rectangles).

GENE (Former names)	Function	GENE (Former names)	Function
GAG	Core Proteins	VIF (SOR, A P', Q)	Infectivity factor
POL	Enzymes	VPR (R)	not known
ENV	Envelope protein	VPU	not known
TAT (TAT-3, TA)	Positive regulator	NEF (3' ORF, B, E', F)	Negative regulator

from West Africa which are only weakly reactive with HIV-1 antisera. Variations occur within HIV-1 and 2 types. It is likely that more HIV types may be identified.

Cell tropism

The receptors for the surface spike proteins of HIV is the CD_4 antigen and hence the virus may infect all cells bearing CD_4. These are principally the T_4 (helper/inducer) the lymphocytes in which the virus induces a lytic infection. Some other immune cells also possess CD_4 on the surface and so are susceptible to infection. Thus about 5–10 per cent of B lymphocytes and 10–20 per cent of monocytes and macrophages, including specialised macrophages such as alveolar macrophages in lungs and Langerhans cells in the dermis are susceptible. Glial cells and microglia in the central nervous system are also found infected.

Resistance

HIV is not a hardy virus. It is thermolabile, being inactivated in 30 minutes at 56°C and in less than a second at 100°C. At room temperature (20°–25°C), it may survive for about seven days. It withstands lyophilisation. The virus can be inactivated by heating lyophilised Factor VIII at 68°C for 72 hours and liquid plasma at 60°C for 10 hours.

HIV is susceptible to common disinfectants. — 70% ethanol, 35% isopropyl alcohol, 5% formaldehyde, 3% hydrogen peroxide, 0.5% lysol, 2.5% Tween – 20. Standard washing procedures with detergents and hot water are adequate for decontaminating clothes and household utensils. Bleaching powder or household bleach (0.2% sodium hypochlorite) is effective for surface decontamination. For treatment of contaminated medical instruments, a 2% solution of glutaraldehyde is useful.

Animal Immunodeficiency viruses

A number of lymphotropic lentiviruses resembling HIV have been isolated from animals. A disease resembling AIDS in macaque monkeys (simian AIDS or SAIDS) is caused by a retrovirus which has been named '*Simian Immunodeficiency Virus* or SIV'. SIV causes natural infection in mangabeys, vervets and African green monkeys, but the infection is asymptomatic in these species. SIV is antigenically related to HIV-2.

A lymphotropic retrovirus causing feline AIDS-like syndrome (FAIDS) has been isolated from cats in the U.S.A. This has been called '*Feline T Lymphotropic Virus* or FTLV'. A bovine lentivirus has been reported that causes a degenerative wasting disease with CNS defects.

HIV-I does not produce disease in any animal. Primates such as chimpanzees and baboons can be infected experimentally leading to antibody response, but no illness. HIV-2 has been reported to infect rhesus monkeys.

Pathogenesis

In an infected individual, HIV can be isolated from blood, lymphocytes, cell-free plasma, semen, cervical secretions, saliva, tears, urine and breast milk (Table 62.3).

Infection is transmitted when the virus enters the blood or tissues of a person and comes into contact with a suitable host cell — principally the T_4 lymphocytes. Infection is likely to result more often following the introduction of HIV infected cells (as in blood transfusion or sexual contact) than of cell-free virus (as in injection of blood products).

A double stranded DNA transcript of the viral DNA is integrated into the genome of the infected cell causing a latent infection. From time to time, lytic infection is initiated releasing progeny virions which infect other cells. The long and variable incubation period of HIV infection is because of the latency.

The primary pathogenic mechanism in HIV infection is the damage caused to the T_4 lymphocyte. T_4 cells decrease in numbers and the T_4:T_8 helper:suppressor) cell ratio is reversed. Depression of T_4 cell function is caused not entirely by cell lysis, because only a small proportion of the cells are found infected with HIV in AIDS, perhaps only one in 10^4 or 10^5 cells. Viral infection can suppress the function of infected cells without causing structural damage. Infected T_4 cells do not appear to release normal amounts of Interleukin-2, gamma interferon and other lymphokines. This has a marked dampening effect on cell mediated immune response.

TABLE 62.3

Sites from which HIV can be isolated

Blood	A. CD_4 + cells T_4 Lymphocytes (Also some monocytes, B-lymphocytes).
	B. Cell-free virions present in plasma.
Semen Cervical fluid	Lymphocytes and cell free.
Saliva Breastmilk C.S.F.	Lymphocytes
Tears	? Cell free
Skin	Langerhan's cells.
Lungs	Alveolar macrophages
CNS	Glial cells, microglia (macrophages)

Though the major damage is to cellular immunity, humoral mechanisms also are affected. Helper T cell activity is essential for optimal B cell function, particularly in responding to thymus dependent antigens. AIDS patients are unable to respond to new antigens. An important feature in HIV infection is the polyclonal activation of B lymphocytes leading to hypergammaglobulinaemia. All classes of immunoglobulins are involved, but levels of IgG and IgA are particularly raised. In infants and children, IgM levels also are elevated. The hypergammaglobulinaemia is more a hindrance than a help because it is composed mainly of 'useless immunoglobulin' to irrelevant antigens and also autoantibodies. This may also be responsible for allergic reactions due to immune complexes (type 3 hypersensitivity).

Monocyte-macrophage function is also affected apparently due to the lack of secretion of activating factors by T_4 lymphocytes. As a result, chemotaxis, antigen presentation and intracellular killing by monocytes/macrophages are diminished. The activity of NK cells and cytotoxic T lymphocytes is also affected.

The principal immunological abnormalities seen in HIV infection are listed in Table 62.4.

Clinical manifestations in HIV infections are due not primarily to viral cytopathology, but are secondary to the failure of immune responses. This renders the patient susceptible to opportunist infections and malignancies. An exception to this may be the dementia and other degenerative neurological lesions seen in AIDS. It has been suggested that these may be due to the direct effect of HIV on the central nervous system.

ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

Clinical features of HIV Infection

AIDS is only the last stage in the wide spectrum of clinical features in HIV infection. The Center for Disease Control (USA) has classified the clinical course of HIV infection under various groups (Table 62.5).

The natural evolution of HIV infection can be considered in the following stages.

TABLE 62.4
Immunological abnormalities in HIV Infection

I. Features that characterise AIDS:
1. Lymphopenia.
2. Selective T cell deficiency - Reduction in number of T_4 (CD_4) cells: Inversion of T_4:T_8 ratio.
3. Decreased delayed hypersensitivity on skin testing.
4. Hypergammaglobulinaemia - predominantly IgG and IgA; and IgM also in children. Infants children IgM ↑
5. Polyclonal activation of B cells and increased spontaneous secretion of Ig.

II. Other consistently observed features:
1. Decreased in *vitro* lymphocyte proliferative response to mitogens and antigens.
2. Decreased cytotoxic responses by T cells and NK cells.
3. Decreased antibody response to new antigens.
4. Altered monocyte/macrophage function.
5. Elevated levels of immune complexes in serum.

TABLE 62.5

Summary of classification system for HIV Infection (Centre for Disease Control, U.S.A.)

Group I	Acute infection
Group II	Asymptomatic infection
Group III	Persistent generalised lymphadenopathy
Group IV	Other diseases
Subgroup A–	*Constitutional disease* - ARC
Subgroup B–	Neurologic diseases
Subgroup C–	Secondary infectious diseases
Category C-1–	Specified infectious diseases listed in the CDC surveillance definition for AIDS, such as *P. carinii* pneumonia, cryptosporidiosis, toxoplasmosis, generalised strongyloidiasis, cryptococcosis, CMV or herpes diseases, etc.
Category C-2–	Other specified secondary diseases, such as oral hairy leukoplakia, salmonella bacteremia, nocardiosis, tuberculosis, thrush
Subgroup D–	Secondary cancers, such as Kaposi's sacroma, Lymphomas
Subgroup E–	Other conditions

1. Acute HIV infection: Within a few weeks of infection with HIV, about 10–15 per cent of persons experience low grade fever, malaise, headache, lymphadenopathy, sometimes with rash and arthropathy resembling glandular fever. Rarely, there may be acute encephalopathy. Spontaneous resolution occurs within weeks. Tests for HIV antibodies are usually negative at the onset of the illness, but become positive during its course. Hence this syndrome has been called 'seroconversion illness', though in the majority of those infected with HIV, seroconversion occurs without any apparent illness. HIV antigenaemia (p24 antigen) can be demonstrated at the beginning of this phase. The pathogenesis of seroconversion illness is believed to be due to immune complexes as well as to direct effects of viral multiplication.

2. Asymptomatic infection: All persons infected with HIV, whether they experience seroconversion illness or not, pass through a phase of symptomless infection, lasting for several months or years. They show positive HIV antibody tests during this phase and are infectious. In some, the infection may not progress any further, while in others it may lead to full blown AIDS, either directly or through cytopaenias, minor opportunistic infections, persistent generalised lymphadenopathy or AIDS-related complex (ARC) as described below.

3. Persistent Generalised Lymphadenopathy (PGL): This has been defined as the presence of enlarged lymph nodes, at least 1.0 cm, in diameter, in two or more noncontiguous extra-inguinal sites, that persist for at least three months, in the absence of any current illness or medication that may cause lymphadenopathy. This by itself is benign, but a proportion of the cases may progress to ARC or AIDS.

K.M.

4. AIDS-Related Complex (ARC): This group includes patients with considerable immunodeficiency, suffering from various constitutional symptoms or having minor opportunistic infections. The typical constitutional symptoms are fatigue, unexplained fever, persistent diarrhoea and marked weight loss — more than 10 per cent of body weight. The common opportunistic infections are oral candidiasis, herpes zoster, hairy cell leucoplakia, salmonellosis or tuberculosis. Generalised lymphadenopathy and splenomegaly are usually present. ARC patients are usually severely ill and many of them progress to AIDS in a few months.

5. AIDS: This is the end stage disease representing the irreversible breakdown of immune defence mechanisms, leaving the patient prey to progressive opportunistic infections and malignancies.

The clinical severity of AIDS varies with the type of infection or malignancy present. In early AIDS, many patients are ill only during episodes of infection, which may respond to treatment. Between episodes they may be relatively well and able to resume normal life. Patients with Kaposi's sarcoma are less ill than those with other malignancies. The illness inexorably progresses and death ensues in months or years. According to the system most affected, patients present with various complaints, some of which are as follows:

a. Respiratory: The commonest presentation is with increasing dry cough, dyspnoea and fever. In the USA and other western countries, the usual pathogen is *P. carinii*, but in the developing countries it is *M. tuberculosis* or an atypical mycobacterium such as *M. avium intra-cellulare*. Pneumonia may also be viral (CMV) or fungal (histoplasma, cryptococcus).

b. Gastrointestinal: The mouth is often involved in AIDS, with thrush, herpetic stomatitis, gingivitis, hairy leukoplakia or Kaposi's sarcoma. Dysphagia may be due to oesophageal candidiasis. Diarrhoea, abdominal pain and distension are common. A characteristic intestinal pathogen in AIDS is cryptosporidium. Salmonellae, mycobacteria, isospora, CMV or adenoviruses also frequently cause intestinal infections. Systemic strongyloidosis may occur. Chronic colitis is common in male homosexuals ('gay bowel syndrome') from which amoeba, giardia and a host of diarrhoeagenic bacteria have been reported.

c. Central nervous system: The typical CNS opportunist infections are toxoplasmosis and cryptococcosis. Infections are also seen with CMV, herpes simplex, papovaviruses, mycobacteria, aspergillus and candida. Lymphomas of the central nervous system are common.

d. Malignancies: Kaposi's sarcoma is the characteristic lesion seen in male homosexuals. It is an indolent multifocal non-metastasising mucosal or cutaneous tumour, probably of endothelial origin. The other tumours commonly seen are lymphomas, both Hodgkin and non-Hodgkin types.

e. Cutaneous: Besides Kaposi's sarcoma, herpes lesions, candidiasis, xeroderma, seborrhoeic dermatitis, tinea, folliculitis, impetigo and molluscum contagiosum are common cutaneous lesions.

6. Dementia: HIV may cause direct cytopathogenic damage in the central nervous system. It can cross the blood brain barrier and cause encephalopathy leading to loss of higher functions, progressing to dementia.

7. Paediatric AIDS: About half the number of babies born to infected mothers are infected with HIV. Many of them may not survive for a year. Children may also acquire the infection from blood transfusion or blood products.

There are many differences between adult and paediatric AIDS. Children develop humoral immunodeficiency early, leading to recurr rial infections. Failure to thrive, chr rhoea, lymphadenopathy, tuberculosi portunist bacterial infections are com ifestations in paediatric AIDS. Lymph

terstitial pneumonia is seen exclusively in children while Kaposi's sarcoma, toxoplasmosis and cryptococcosis are less common than in adults.

Laboratory diagnosis

Laboratory procedures for diagnosis of HIV infection include tests for immunodeficiency as well as specific tests for HIV.

A. *Immunological tests:* The following parameters help to establish the immunodeficiency in HIV infection.

1. Total leucocyte and lymphocyte count to demonstrate leucopenia and a lymphocyte count usually below 2000/c.mm.
2. T cell subset assays. Absolute T_4 cell count will be usually less than 200/c. mm. T_4:T_8 cell ratio is reversed.
3. Platelet count will show thrombocytopaenia
4. Raised IgG and IgA levels.
5. Diminished CMI as indicated by skin tests

B. *Specific tests for HIV infection:* These include demonstration of HIV antigens and antibodies, and isolation of the virus.

1. ANTIGEN DETECTION

The time course of appearance of detectable antigen and antibodies after HIV infection has not been adequately defined. Following a single massive infection, as by blood transfusion, the virus antigen (p24) may be detectable in blood after about two weeks. IgM antibodies appear in about 4-6 weeks, to be followed by IgG antibodies (Fig. 62.3; Table 62.7).

If the infecting dose is small, as following a needle stick injury, the process may be considerably delayed. The appearance of p24 antigenaemia and viraemia followed by the early antibody response coincide with the acute or seroconversion illness. Afterwards, the p24 antigen disappears from circulation and remains absent during the long asymptomatic phase, to reappear only when clinical disease sets in. Tests for antigen detection are available only in specialised laboratories and therefore not used routinely.

2 VIRUS ISOLATION

Once infected with HIV, a person remains infected for life. The virus is present in circulation

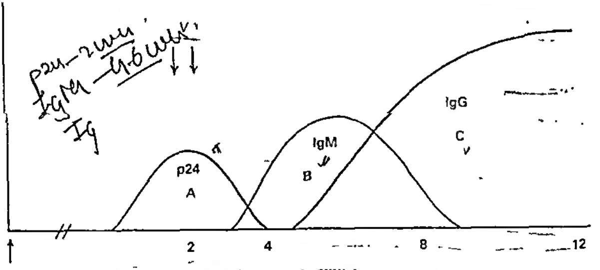

Fig. 62.3 Sequences of appearance of antigens and antibodies after HIV infection.
↑ exposure A. antigen B and C antibody. 2,4,8 and 12 express. weeks after exposure.

TABLE 62 6

Opportunistic Infections and malignancies commonly associated with HIV Infection (in developed countries)

I.	Parasitic –
	1. *Pneumocystis carinii* pneumonia
	2. Toxoplasmosis
	3. Cryptospondiosis
	4. Isosporiasis
	5. Generalised strongyloidiasis
II.	Mycotic –
	1. Candidosis
	2. Cryptococcosis
	3. Aspergillosis
	4. Histoplasmosis
III.	Bacterial –
	1. Mycobacterial infections – Tuberculosis and non-tuberculous infections
	2 Salmonellosis
	3. Campylobacter infection
	4. Nocardia and actinomycetes
	5. Legionellosis
IV.	Viral –
	1. CMV
	2. Herpes simplex.
V.	Malignancies –
	1. Kaposi's sarcoma.
	2. Lymphomas - Hodgkin and non-Hodgkin types.

and body fluids, mostly within lymphocytes but some also cell-free. Virus titres are high early in infection about a week before antibodies start appearing. Antibodies do not neutralise the virus and the two can coexist in the body. During the phase of asymptomatic infection, virus titre is low and may not be detectable, but when clinical AIDS sets in, the titre rises once again. An infected person may therefore be infectious throughout, but the infectivity is highest in the early phase of infection (when the antibody tests are negative and the case may not be detected in screening tests) and again when he becomes clinically ill.

Though the virus is present in many parts of the body, it is most readily isolated from peripheral lymphocytes. The technique of isolation is by co-cultivation of patient's lymphocytes with uninfected lymphocytes in the presence of Interleukin-2. Viral replication can be detected by demonstration of reverse transcriptase activity as well as antigen in the system. However, viral isolation is not suitable as a routine diagnostic test.

3. ANTIBODY DETECTION

Demonstration of antibodies is the simplest and most widely employed technique for diagnosis of HIV infection. However it needs to be emphasised that it may take several weeks to months for antibodies to appear after infection, and during the later part of this period, the individual may be infectious. For this reason, antibody screening is not totally dependable for spotting

TABLE 62.7
Evolution of serological markers during HIV infection

State of infection	Markers			
	p. 24 antigen	anti-HIV IgG	anti-HIV IgM	Western blot pattern
Early infection	− → +	−	−	−
Acute (sero-conversion) illness	+ → −	− → +	+	Partial: p24 and/or gp120
Carrier, asymptomatic	−	+	−	Full pattern
PGL	+	+	−	Loss of p24/p55
AIDS	+	+	−	Absence of p24: loss of other reactivities

infectious persons, as for example among blood donors. Many instances of HIV transmission by blood transfusion from seronegative donors have been documented. It is stated that following sexual exposure to HIV, antibodies appear within 3–6 months if infection has taken place. Therefore antibody testing after six months may help to identify whether infection has occurred after a single sexual exposure.

Once antibodies appear they increase in titre and broaden in spectrum for the next several months. IgM antibodies disappear in 8–10 weeks while IgG antibodies remain throughout. When immunodeficiency becomes severe following clinical AIDS, some components of anti-HIV antibody (e.g., anti-p24) may disappear.

Serological tests for anti-HIV are of two types— *screening* and *confirmatory* tests. Screening tests possess high sensitivity, a broadly reactive spectrum, are simple to perform and can be automated for handling large numbers of samples at a time. They are not highly specific and may give a few false positive results. All sera positive on screening tests are to be checked by a confirmatory test before the sample is declared as positive. The most widely used screening test is ELISA.

1. **ELISA tests:** Direct solid phase ELISA is the method most commonly used. The antigen obtained from HIV grown in continuous T lymphocyte cell line or by recombinant techniques is coated on microtitre wells or other suitable solid surface. The test serum is added, and if antibody is present, it binds to the antigen. After washing, away the unbound serum, anti-human immunoglobulin linked to a suitable enzyme is added, followed by a colour forming substrate. If the test serum contains anti-HIV antibody, a visible or photometrically detectable colour is formed which can be read visually or by special ELISA readers.

ELISA is simple and relatively inexpensive, but false positive reactions are not uncommon, particularly with sera containing rheumatoid factor or anti-lymphocyte antibodies. Sera stored for long periods contain nonspecific 'sticky' immunoglobulins. Plasma gives more false positive tests than serum, particularly in agglutination tests. Persons from some parts of the world may

show nonspecific reaction due to cross reacting antibodies to locally prevalent non-HIV retroviruses.

Modification of ELISA in which the antibody in test serum either *competes* with enzyme-conjugated anti-HIV antibody, or is *captured* by anti-human immunoglobulin on to solid phase are more specific. Capture ELISA specific for IgM antibody is also available.

2. Fujirebio agglutination test in which antigen coated gelatin particles are agglutinated by antibody is a simple and convenient test, but is also subject to false positive reactions.

3. Karpas' test is a slide immunoperoxidase reaction. HIV infected cells fixed on teflon-coated slide wells are treated first with the test serum and then with horse radish peroxidase labelled anti-human globulin. Finally a suitable substrate is added which gives a colour reaction if the test is positive. The test is simple and inexpensive, but the evaluation is subjective.

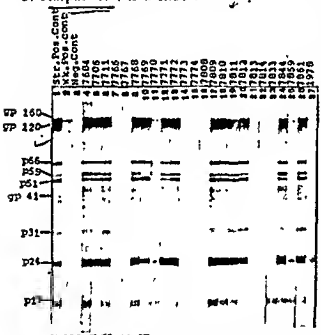

Fig 62.4 Western blot test for HIV antibody. Nitrocellulose strips containing separated HIV proteins are reacted with test (nos 4 to 27) and control strong positive, weak positive and negative (nos. 1 to 3) sera. Antibody bound specifically to HIV proteins is visualised by using goat antihuman immunoglobulin-biotin conjugate, an avidin-horse radish peroxidase conjugate and 4-chloro 1-naphthol substrate. A serum is considered positive if it shows antibody binding to at least 2 gene products of the 3 major HIV genes, namely *gag* (p 17, p 24, p 55), *env* (gp 41, gp 120, gp 160) and *pol* (p 31, p 51 and p 66) Examples of typical results are sera nos 4,5,6 for positive, 7,8 for negative and 11,21 for weak positive reactions [Courtesy - Prof. T. Jacob John, Department of Virology and Immunology, C.M C. Hospital Vellore.]

4. Indirect immunofluorescence again is liable to subjective error. Radio-immunoassay can be used for detecting specific antibodies, as for example anti-p24, but is not widely used.

5. The confirmatory test commonly employed is the Western blot test. In this test, HIV proteins separated according to their electrophoretic mobility (and molecular weight) by polyacrylamide gel electrophoresis are blotted on to strips of nitrocellulose paper. These strips are reacted with test sera and then with enzyme-conjugated anti-human globulin. A suitable substrate is then added which produces a prominent colour band where specific antibody has reacted with the separated viral protein. The position of the band on the strip indicates the antigen with which the antibody has reacted (Fig. 62.4). In a positive serum, bands will be seen with multiple proteins typically with p24 (*gag* gene, core protein), p31 (*pol* gene, reverse transcriptase) and gp41, gp120 or gp160 (*env* gene, surface antigen). However, interpretation becomes difficult when bands appear only at one or two sites, as with p24 or gp120. This may happen in early infection, but may also be *nonspecific*. Western blot is a very useful confirmatory test, but the interpretation remains subjective and demands considerable experience.

Examination of a serum by a battery of tests reduces the chance of nonspecific reactions. A true positive serum will react in all tests, while a false positive would seldom do so. When in doubt, a follow-up testing after 2–3 months would be useful.

Apart from diagnosing HIV infection, the laboratory would be called upon to identify the opportunist infections that are a feature of AIDS. Routine microbiological methods would suffice for this. However, serological diagnosis of infec-

tions may not always be reliable in AIDS as antibody formation may be affected by the immune deficiency.

Applications of serological tests: Serological tests for HIV infection are employed in the following situations.

1. Screening: Screening is defined as the systematic application of HIV testing, whether voluntary or mandatory, to entire populations or selected target groups. Screening of entire populations raises many complex social and psychological issues and is therefore neither feasible nor practicable. But screening of target populations is valuable. As iatrogenic transfer of HIV is an important mode of spread of the infection to unsuspecting recipients, it should be mandatory that all donors of blood, blood products, semen, cells, tissues and organs be screened. As antibody tests are negative during the early stage of HIV infection when the individual is infectious, screening may not detect all dangerous donors, but can still eliminate the large majority of them. A person found positive for anti-HIV antibody should never donate blood or other biological materials. As the infection can be transmitted from mother to baby before, during or after birth, antenatal screening may be considered. Some countries have laws requiring screening of incoming foreigners. In India, foreign students are being screened for anti-HIV antibodies.

2. Sero-epidemiology: Antibody surveys have been most useful in identifying the geographical extent of HIV infection and in other epidemiological studies such as spread of the infection from identified sources.

3. Diagnosis: Serology is almost always positive in persons with clinical features of AIDS. It may however be negative in acute illness and sometimes in the very late cases where the immune system is nonreactive. Routine serology may also be negative when the infection is with a different AIDS virus. For example, HIV-2 infections which are prevalent in Africa are likely to be missed if antibody testing is done with HIV-1 antigen.

Antibody testing may also help to check whether infection has taken place following an exposure, such as sexual contact, blood transfusion or needle stick injury. Serology after two months and, if negative, after six months would be sufficient. If serology is negative six months after exposure, infection is unlikely to have occurred.

4 Prognosis : In a person infected with HIV, loss of detectable anti-p24 antibody indicates clinical deterioration. This is taken as indication for active antiviral treatment. This is also associated with HIV antigenaemia and increased virus titre in circulation.

Epidemiology and prevention

AIDS is a new disease which came to light only in 1981 when it caused outbreaks in the USA. But in retrospect, the virus appears to have been seeded in the United States at least in the mid-1970's and a few unrecognised cases of AIDS had occurred in New York in 1978. The origin of the virus has been the subject of much controversy, reminiscent of the situation five hundred years ago when syphilis was first recognised. It has been suggested that the virus may have originated in Africa, perhaps from a simian immunodeficiency virus and spread to the USA, probably through Haiti. In the permissive American Society of the 1970's, the virus spread widely among male homosexuals and drug addicts, finally to come out into the open as outbreaks in 1981. The virus may have spread to Europe from America, as well as directly from the former African colonies of European nations.

The virus has spread virtually all over the world, though the prevalence rates in different countries vary widely. North America, Brazil, Western Europe, Australia, Central and West Africa have high prevalence, while Eastern Europe and Asia are only sparsely affected. Differences also exist, both in the modes of infection and in clinical manifestations, between the affluent and developing countries. The epidemiology of AIDS has been studied mostly in the

developed nations and only sketchily in the third world.

The modes of transmission of HIV are shown in Table 62.8

HIV is primarily a sexually transmitted infection. In the USA it was transmitted predominantly among male homosexuals. The danger of infection is more for the passive agent because mucosal tears are very frequent during anal intercourse and virus-laden lymphocytes in semen can directly enter through these. In the homosexual males, the relative risk of infection in the various sexual practices has been estimated in the descending order as ano-receptive, oro-receptive, ano-insertive and oro-insertive. One reason for the high incidence of HIV infection in male homosexuals may be the large numbers of sexual partners they are reported to have, sometimes over a thousand. In affluent countries, homosexual and bisexual males are infected far more often than heterosexuals. For this reason infection was found predominently in males and only occasionally in females. However, the situation is very different in Africa and' Asia where males and females are equally affected. In some places, more females are found infected due to the high rate of infection in prostitutes. Transmission in the developing countries is almost always heterosexual and can take place in both directions.

The best method of checking sexual transmission of infection is health education regarding the danger of promiscuity and other high risk activities. Some changes in the life style and sexual attitudes have already taken place in the USA so that incidence in homosexuals has come down. Persons indulging in high risk sexual practices and spouses of infected persons should be counselled regarding 'safer sex' methods. The use of condoms offers considerable protection though not complete protection. The risk of HIV transmission increases with multiple partners.

The second mode of transmission is through blood and blood products. Before the danger of HIV transmission was recognised, many persons had received blood and blood products containing infectious virus. Screening of blood donors is now mandatory in the developed countries, but is not yet practised in the poor nations. Even screening may not completely eliminate the danger as the early infectious case may be missed, but the risk is reduced considerably. In the advanced

TABLE 62.8

Common modes of transmission of HIV

I	Sexual intercourses:
	Anal, vaginal, oral
II	Blood and blood products
	Blood transfusion, Factor VIII etc
III	Tissue and organ donation
	Semen, cornea, bone marrow, kidney etc.
IV	Injections and injuries.
	Shared needles by drug addicts
	Injections with unsterile syringes and needles
	Needlestick and other injuries in health staff
	? Surgical wounds
V	Mother to baby
	Transplacental
	At birth
	After birth
	? Breast milk

countries any person who has indulged in high risk practices is advised not to donate blood. This restriction also applies to donation of semen, cornea, bone marrow, kidney and other organs as infection can be transmitted through any of these. But such restraints may not be enforced in the developing countries, where professional donors constitute a real hazard. Manufacturing processes of blood products have now been modified to eliminate infectious virus.

Contaminated needles can transmit the infection. This is particularly relevant in drug addicts who share syringes and needles. The surest way of countering this would be to wean addicts away from the habit, but this is seldom successful. In some areas where this mode of transmissinn is a serious problem, sterile disposahle syringes and needles have been made available to addicts free of cost to minimise the danger. This mode of infection is more common in the affluent nations than in poor countries where addiction to injectable drugs is rare. More important here is the use of unsterile syringes and needles by doctors and health staff making iatrogenic infection likely. Even in large hospitals, sterilisation methods are often unsatisfactory. In immunisation and family planning camps the same syringe and other instruments are used for many persons. This is a serious threat. Where facilities for sterilisation are not available, the use of disposable needles and equipments may be necessary.

The danger of needlestick injury is present in medical and paramedical personnel, though the chances of infection are much less than with HBV. The risk following needlestick injury or injury with sharp instruments used on seropositive patients has been estimated to be about one per cent. By the middle of 1988, only 15 cases of seroconversion had been documented in health personnel, including laboratory workers, dealing with HIV infected persons, nine following needle stick injury, five after exposures of skin or mucous membrane to infected blood or body fluids, and one in a laboratory worker who sustained a cut while handling the virus. The risk to medical and nursing personnel appears to be minimal provided they take adequate precautions. But considering the unsatisfactory asepsis and hygiene in many hospitals in poor countries, the risk may be real. Medical and paramedical staff need to be educated on caring for patients infected with HIV. Guidelines should be laid down for hospitals regarding the management of AIDS patients. They can be treated in any well organised hospital in the same manner as any other highly infectious disease. Gloves should be worn while handling patients, and protective clothing and spectacles used when there is a danger of splashing of blood or fluids. Soiled materials should be sterilised before disposal. Blood or other fluids splashed on the skin, mouth or eyes should he washed thoroughly. Skin puncture should be encouraged to bleed and then washed.

Transmission of infection from mother to bahy can take place before, during or after birth. As infection occurs in ahout half such babies, infected women should be informed of the danger and advised against pregnancy. HIV may be present in breast milk and may rarely be transmitted through breast feeding.

Normal social and domestic contact does not transmit the infection. Shaking hands, hugging, putting cheeks together or dry kissing is safe. There has been no confirmed case of transmission through saliva, though the virus may be present in the saliva of infected persons. 'Wet kissing' is considered risky. Sharing rooms, bathrooms, cooking and eating facilities are not considered dangerous. There is no evidence that mosquitoes, bed bugs or other blood sucking insects can transmit the virus. Infection is not transmitted through air, food, water or fomites.

AIDS in the developing countries differs from the disease in the Western countries clinically also. In Africa, the major manifestation is pronounced wasting so that it has been called the 'slim disease'. The high prevalence of tuberculosis and parasitic infections complicate the clinical picture.

India is one of the countries where HIV infection has come in late and spread slowly. From the available information, transmission has been

mainly heterosexual, involving prostitutes. Infection through blood transfusion has also occurred. The first case of AIDS identified in India had acquired the infection abroad, but indigenous cases have since been observed and are on the increase. Surveillance units have been set up in different parts of the country.

Specific prophylaxis of HIV infection at present depends on health education, identification of sources and elimination of high risk activities. Great efforts are being made to develop a vaccine, but no headway has been made so far. In the case of some animal lentiviruses, vaccines not only do not protect, but may actually worsen the outcome of infection. Prospects for a vaccine in the near future are dim.

Treatment

Approaches to the treatment of AIDS include: 1) the treatment and prophylaxis of infections and tumours. 2) general management, 3) immunorestorative measure, and 4) specific anti-HIV agents.

Prompt diagnosis and appropriate treatment of opportunistic infections and tumours in the early stage of AIDS can be very useful and the patient may be able to resume normal life in between episodes of illness. General management of the patient requires the understanding and cooperation of the health staff in the hospital and of relatives at home. Groundless fears about imaginary risks have to be allayed and reassurance given that the patient can be kept at home or treated in the hospital without danger to contacts, if proper precautions are taken.

Steps at immunorestorative therapy such as administration of interleukin-2, thymic factors, leucocyte transfusion and bone marrow transplantation have not been very helpful.

A large number of antiviral drugs, including alpha interferon, ribavirin, suramin, foscarnet have been tried in AIDS patients and abandoned due to high toxicity and insufficient benefit. Only one drug, Zidovudine (azidothymidine, AZT) has been licensed for use in AIDS. This can be given orally and is able to cross the blood brain barrier. Multicentric field trials have shown significant clinical and immunological benefit from the use of this drug. Though many other compounds are being tested, Zidovudine is the only effective therapeutic agent against HIV available at present.

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63 Normal Microbial Flora of the Human Body

Man, like other animals, harbours a wide array of microorganisms either on or in his body. The normal microbial flora are more or less constant for each species and are broadly divided into residents and transients. The former constitute a constant population which cannot be completely removed permanently, while the latter vary from time to time and are impermanent. The residents prevent permanent colonisation of the body by other organisms. A knowledge of the normal flora of the body is essential to an understanding of the interaction of man and his pathogen laden environment. The normal microbial flora play an important role in body economy. They can 1) become pathogenic when host defences falter, 2) prevent or interfere with colonisation/invasion of the body by pathogens, 3) raise the overall immune status of the host against pathogens having related or shared antigens, and 4) cause confusion in diagnosis due to their ubiquitous presence in the body and their resemblance to some of the pathogens. Members of the normal flora form part and parcel of the host and include saprophytes, commensals, facultative pathogens and true pathogens.

The microflora of the intestinal tract synthesise Vitamin K and several B vitamins which supply on occasion the body's needs. The antibiotic substances produced by some, e.g., colicins, have a harmful effect on pathogens. The endotoxins liberated by them may help the defence mechanism of the body by triggering the alternative complement pathway, as long as they are not produced in excessive amounts.

On the contrary, the opportunist pathogens among them cause disease when the body's defence mechanisms fail. Their abnormal multiplication can cause diseases such as enteritis and endotoxic shock. Penicillinase producing organisms can aggravate infection by interfering with therapy. Certain streptococci of the mouth cause dental caries.

In environments laden with pathogens — as hospitals — a shift in the normal flora of the individuals there can occur, for example, increase in carriage of antibiotic resistant staphylococci. It has also been shown that such people can be recolonised with penicillin sensitive staphylococci of strain 502 A which are harmless and thus overcome the damage done. When large numbers of people congregate from different parts of the country as in army camps, the new recruits experience increased colonisation rates of *Neisseria meningitidis* and *Group A Streptococcus* and viruses such as rhinoviruses and adenoviruses resulting sometimes in epidemics.

NORMAL FLORA OF THE SKIN

The human skin is constantly and continuously bombarded by organisms present in the environment. It is also contaminated by one's own secretions and excretions, the extent depending on the individual's personal hygiene. The flora depend on the area of the body, the clothing one wears, one's occupation and environment. Transient microflora tend to occur more frequently on the skin.

Cultures from the skin have frequently demonstrated diphtheroids; staphylococci (aero-

bic and anaerobic); Gram positive aerobic spore bearing bacilli; *Str. viridans; Str. faecalis;* Gram negative bacilli such as *E. coli,* Proteus, and other intestinal organisms; mimieae; mycobacteria (nonpathogenic); *Candida albicans;* cryptococci and *Pityrosporum ovale.*

Often the skin of the face, neck, hands and buttocks carries pathogenic haemolytic streptococci and staphylococci. Penicillin resistant staphylococci, are seen in individuals working in hospitals.

Hair frequently harbours *Staph. aureus* and forms a reservoir for cross infection.

NORMAL FLORA OF THE CONJUNCTIVA

The conjunctiva is relatively free from organisms due to the flushing action of tears. The predominant organisms of the eye are diphtheroids (*Corynebacterium xerosis*), Moraxella species, staphylococci and nonhaemolytic streptococci.

NORMAL FLORA OF THE NOSE, NASOPHARYNX AND ACCESSORY SINUSES

The floor of the nose harbours corynebacteria, staphylococci and streptococci. *Haemophilus* species and *Moraxella lacunata* may also be seen.

The nasopharynx of the infant is sterile at birth but, within 2–3 days after birth, acquires the common commensal flora and the pathogenic flora carried by the mother and the attendants. The nasopharynx can be considered the natural habitat of the common pathogenic bacteria which cause infections of the nose, throat, bronchi and lungs. Certain Gram negative organisms from the intestinal tract such as *Pseudomonas aeruginosa, E. coli,* paracolons and Proteus are also occasionally found in normal persons. After penicillin therapy, they may be the predominant flora.

NORMAL FLORA OF THE MOUTH AND UPPER RESPIRATORY TRACT

The mouth contains a plethora of organisms — pigmented and nonpigmented micrococci, some of which are aerobic, Gram positive aerobic spore bearing bacilli, coliforms, Proteus and lactobacilli. The gum pockets between the teeth, and crypts of the tonsils have a wide spectrum of anaerobic flora — anaerobic micrococci, microaerophilic and anaerobic streptococci, vibrios, fusiform bacilli, corynebacterium species, actinomyces, leptothrix, mycoplasma, neisseria, bacteroides are all found in varying extent. Among fungi, Candida and geotrichum have been reported.

The mouth of the infant is not sterile at birth. It generally contains the same types of organisms in about the same relative numbers as those present in the mother's vagina, i.e., a mixture of micrococci, streptococci, coliform bacilli and Doderlien's bacilli. These organisms diminish in number during the first two to five days after birth and are replaced by the types of bacteria present in the mouth of the mother and nurse.

Within 12 hours after birth alpha haemolytic streptococci are found in the upper respiratory tract and become the dominant organism of the oropharynx and remain so for life. In the pharynx and trachea, flora similar to that of the mouth establish themselves. Few bacteria are found in normal bronchi. Smaller bronchi and alveoli are normally sterile.

NORMAL FLORA OF THE INTESTINAL TRACT

In 80–90 per cent newborn infants the meconium is sterile but in 10–20 per cent a few organisms, probably acqured during labour, may be present. In all cases within 4–24 hours of birth an intestinal flora is established partly from below and partly by invasion from above. In breast fed children the intestine contains lactobacilli (*L. bifidus* constituting 99 per cent of total organisms in the faeces), enterococci, colon bacilli and staphylococci. In artifically fed (bottle fed) children *L. acidophilus* and colon bacilli and in part by enterococci, Gram positive aerobic and anaerobic bacilli. With the change of food to the adult pattern, the flora change. Diet has a marked infuence on the relative composition of the intestinal and faecal flora.

In the normal adult, the microorganisms on the surface of the oesophageal wall are those swallowed with saliva and food. Because of the low pH of the stomach, it is virtually sterile except soon after eating. In patients with carcinoma of the stomach or achlorhydria or pyloric obstruction, there is proliferation of Gram positive cocci and bacilli.

The number of bacteria increases progressively beyond the duodenum to the colon, being comparatively low in the small intestine. In the adult duodenum there are 10^3–10^6 bacteria per gram, in the jejunum and proximal ileum 10^5–10^8 bacteria per gram, and in the lower ileum and caecum 10^8–10^{10} bacteria per gram of contents. In the duodenum and upper ileum, lactobacilli and enterococci predominate, but in the lower ileum and caecum the flora resemble the faecal flora. There are about 10^{11} bacteria per gram of contents in the colon and rectum, constituting 10–20 per cent of the faecal mass. In the adult normal colon, the resident bacterial flora are mostly (96–99 per cent) anaerobes — anerobic streptococci, anaerobic lactobacilli, clostridia, and bacteroides and about 1–4 per cent aerobes — enterococci, coliforms, and small numbers of Proteus, Pseudomonas, lactobacilli, mycoplasma, Candida and others.

NORMAL FLORA OF THE GENITOURINARY TRACT

Mycobacterium smegmatis, a harmless commensal, is found in the smegma of the genitalia of both males and females. This may, by its presence in the voided specimens of urine, cause confusion. From apparently normal men, aerobic and anaerobic bacteria were cultured from a high proportion, including lactobacilli, *Gard. vaginalis*, alpha haemolytic streptococci and *Bacteroids* species *Chlam. trachomatis* was found in three per cent and *Ureaplasms urealyticum* in 59 per cent of them. The female urethra is either sterile or contains a few Gram positive cocci.

The vulva of the newborn child is sterile but after 24 hours it acquires a varied flora of nonpathogenie organisms from the skin, vagina and intestines. The nature of the flora in the vagina depends on the pH of its secretions and its enzyme content. At birth the vagina is sterile. In the first 24 hours it is invaded by micrococci, enterococci and diphtheroids. In two to three days, the maternal oestrin induces glycogen deposition in the vaginal epithelium. This facilitates the growth of a lactobacillus (Doderlien's bacillus) which produces acid from glycogen, and the flora for a few weeks is similar to that of the adult. After the passively transferred oestrin has been eliminated in the urine, the glycogen disappears, along with Doderlien's bacillus and the pH of the vagina becomes alkaline. This brings about a change in the flora to micrococci, alpha and nonhaemolytic streptococci, coliforms and diphtheroids. At puberty, the glycogen reappears and the pH changes to acid due to the metabolic activity of Doderlien's bacilli, *E. coli* and yeasts. This change probably helps in prevention of colonisation by possible harmful mieroorganisms. During pregnancy there is an increase in *Staphylococcus albus*, Doderlien's bacilli and yeasts. Occasionally other members of the intestinal flora may be present. After menopause, the flora resembles that found before puberty. The normal vaginal flora often includes clostridia, listeria, anaerobic streptococci, mimeae, mycoplasma, *Gardnerella vaginalis*, neisseriae and Treponema species.

BACTERIA IN THE BLOOD AND TISSUES

The commensals from the normal flora of the mouth, nasopharynx and intestinal tract may get into the blood and tissues. They are usually quickly eliminated by the normal defence mechanisms of the body. Occasional isolation of diphtheroids or nonhaemolytic streptococci from normal and abnormal lymph nodes may be those which escaped elimination. Unless the organisms of doubtful pathogenicity are isolated more than once in serial blood cultures, they have little significance.

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64 Bacteriology of Water, Milk and Air

BACTERIOLOGY OF WATER

Water meant for human consumption must be free from chemical substances and microorganisms which might be dangerous to the health of the community. Drinking water supplies should, over and above being safe, be also pleasant to drink, i.e., cool, clear, colourless and devoid of disagreeable taste or smell. The situation, the construction and the maintenance of the water supply, its storage and distribution system must exclude any possible pollution of the water.

That all waters are liable to pollution must be constantly kept in mind. Both chemical and bacteriological examination of the water supply from the source to the consumer should be regularly and systematically done, though in several cases chemists and bacteriologists may disagree. The aim of microbiological examination of water supplies is to detect whether pollution of the water by pathogenic organisms has occurred or not. Though it would be ideal to look for the possible pathogens themselves it is not practicable since they are usually few and far outnumbered by nonpathogenic organisms and hence the methods to detect them are costly in time and money. Therefore we look for indicators of human/animal pollution, i.e., intestinal organisms. The organisms most commonly used as indicators of pollution are *E. coli* and the coliform group as a whole. *Streptococcus faecalis* and *Clostridium welchii* are also searched for 1) when the nature of pollution is doubtful, 2) when water is examined at infrequent intervals, and 3) when a new source of water supply is being considered.

Bacterial flora in water

The bacteria found in water can be conveniently divided into three groups:

1. Natural water bacteria: The organisms commonly found in water free from gross pollution are included in this group. Chromogenic and nonchromogenic cocci, *Sarcinae*, fluorescent, chromogenic and nonchromogenic bacilli are found. The fluorescent bacilli are *Ps. fluorescens*, the chromogenic bacilli are *Serratia*, *Flavobacterium* and *Chromobacterium violaceum*. The nonchromogenic bacilli belong to the *Achromobacter* group.

2. Soil bacteria: These get washed into the water during heavy rains. Most belong to the group of aerobic spore bearing bacilli. *Klebs. aerogenes* and *Klebs. cloacae* which may be found on grains, plants and decaying vegetation are other aerobic nonsporing bacilli which may also be included in this group for convenience. Nitrifying bacteria, streptomyces and certain fungi may also be isolated from waters. The latter two may impart an earthy odour and taste to the water on occasions. Polythene pipeline favours the growth of bacteria, some of which produce copious quantities of slime often leading to blockage.

3. Sewage bacteria: Many of the organisms in this group are normal inhabitants of the intestine of man and animals. Others live mainly on decomposing organic matter of either animal or vegetable origin. Sometimes pathogenic organisms are included. The bacteria in this group are:

1. Intestinal bacteria: *E. coli* group. *Streptococcus faecalis*, *Cl. welchii*, pathogenic organisms such as *S. typhi*, and *V. cholerae*.
2. Sewage bacteria: Proteus, *Cl. sporogenes*.

Factors determining the kinds and number of bacteria in water

1. Type of water: Depending on the source, waters are divided into surface and deep. Surface waters are those that have not been filtered through any considerable thickness of soil. They are exposed to contamination from dust, soil, sewage, industrial waste and other decomposing organic matter and hence may contain large numbers of bacteria, many of which may be of intestinal origin. Deep waters are those which have percolated through several hundred feet of porous layers of soil and are generally pure. Even when bacteria are found in them, they are generally of a harmless type.

2. Organic matter: When these are plentiful, organisms abound and as they become scarce, organisms decrease correspondingly.

3. Temperature: The effect of temperature depends to a large extent on the amount of organic matter present. When organic matter content is high, a rise of temperature favours multiplication of bacteria with consequent increase in their numbers. Generally, a low temperature favours the survival of bacteria.

4. Light: The effect is only theoretical. Actinic rays of the sun can be bactericidal, provided the water is clear and static and even then possibly only to a depth of five feet.

5. Acidity: It is believed that acidity of water has a bactericidal action and that it plays a considerable part in the purification of some waters.

6. Salinity: A high salinity has a deleterious effect on most bacteria.

7. Protozoa: Protozoa, by ingesting and destroying bacteria, play a part in keeping the bacterial population down.

8. Rainfall: Early in the rainy season they wash down into the water sources large numbers of bacteria from the soil. But later, when the water level rises, they dilute out the bacterial population.

9. Storage: Storage of water in a reservoir brings down the bacterial content. This may be due to 1) sedimentation. Organisms stick to suspended matter and sink leaving the supernatant clear, 2) equalisation, when water from different sources and hence differing bacterial content get collected in the same reservoir, and 3) devitalisation due to decrease in food supply and action of protozoa.

Pathogenic organisms survive longer in pure than in impure water. Therefore pollution of purified water in reservoirs and water mains have to be very stringently guarded against.

Bacteriological examination

1. Sampling: The frequency of bacteriological examination and the location of sampling points should be such as to enable the bacterial quality of water supply to be properly controlled. The points from which samples are to be taken and their frequency should be decided in consultation with an expert sanitary adviser. The bacteriological examinations should be carried out in authorised laboratories.

The bacteriological examination of disinfected water as it enters the distribution system from such treatment point should be carried out at least once a day. With small supplies, serving a population of 10,000 or less, daily sampling may be impracticable and we should rely on proper control of disinfectant dosage and bacteriological examination at weekly intervals. Supplies that do not require disinfection, but are chlorinated as a precautionary measure, may not need daily bacteriological tests. The following tables give gui-

TABLE 64 1

Maximum intervals between successive samples of nondisinfected water entering the distribution system

Population served	Maximum interval between successive samples
Less than 20,000	1 month
20,000 to 50,000	2 weeks
50,000 to 1,00,000	4 days
More than 1,00,000	1 day

TABIE 64 2

Maximum intervals between successive samples and minimum number of samples to be taken from the distribution system whether the water has been subjected to disinfection or not

Population served	Maximum interval between successive samples	Minimum number of samples to be taken from the whole distribution system each month
Less than 20,000	1 month	One sample per 5,000 population per month
20,000 to 50,000	2 weeks	
50,001 to 1,00,000	4 days	
More than 1,00,000	1 day	One sample per 10,000 population per month

dance as to the number and frequency of samples to be collected.

Both the above criteria should be satisfied in every distribution system.

It is far more important to examine numerous samples by a simple test than occasional samples by a more complicated test or series of tests, as one can never predict when pollution may occur. In unfavourable circumstances or in the event of an epidemic or immediate danger of pollution or when more stringent control is necessary, as for example with water supplies to dairies or food processing plants, much more frequent bacteriological examination is required.

2. *Collection, transport and storage of samples for bacteriological examination*: The sample should be representative of the water, it is desired to examine. All care should be taken to avoid contamination. When several samples are being collected on the same occasion from the same source, the sample for bacteriological examination should be collected first. Sterilised glass bottles provided with ground glass stoppers should be protected by kraft paper. If the water to be sampled contains or is likely to contain traces of chlorine or chloramine or ozone, a sufficient quantity of sodium thiosulphate should be added to the sampling bottles before sterilisation.

The sampling bottle should not be opened until it is required for filling. It should be held near its bottom. It should be filled without rinsing and the

stopper should be immediately replaced. If water is collected from a tap, it should be cleaned and flamed and the water allowed to run from the tap for at least two minutes before the sample is collected.

When collecting samples from rivers, streams, lakes or reservoirs, it is not desirable to take samples from too near the bank or too far from the point of draw off. The bottle should be held near the bottom, plunged neck downwards below the surface, turned so that the neck points slightly upwards with the mouth facing the direction of the current. If no current exists we should push the bottle horizontally forward. In the case of shallow wells where the method detailed cannot be followed, the bottle should be weighted and lowered into the water. If water has to be collected from a depth, a string or cord is attached to the neck and weighted, and the bottle lowered to the desired depth. The stopper should have a removal and replacing mechanism — a string or wire tied to it.

If water is to be taken from a pump, the pump outlet should be sterilised after pumping water to waste for five minutes, then more water pumped to waste and then the sample collected avoiding contamination.

Water should be examined as early as possible after collection, preferably within one hour but the interval between collection and examination should never exceed twentyfour hours. Where delay is expected, the sample can be filtered using millipore membranes at the site or in a nearby laboratory. The membrane should be placed after filtration, on an absorbent pad saturated with transport medium in a Petri dish and then sent to the laboratory. Delays of upto three days make little difference to counts of coliform organisms and *E. coli*.

3. The examination: A full bacteriological examination should be carried out 1) when a new source of water is being selected, and 2) when circumstances warrant its need. This consists of 1) colony counts of microorganisms on nonselective media, 2) count of coliform organisms and *E. coli*, 3) examination for faecel streptococci and *Clostridium welchii*, and 4) examination for 'nuisance' bacteria such as green-fluorescent pseudomonas (in special circumstances).

Organisms indicative of faecal pollution: Contamination by sewage or by human and animal faeces is the greatest danger to drinking water supplies. If such contamination is of recent origin and it has also been due to cases of carriers of enteric diseases, the water may contain the live organisms causing these diseases. The use of such water may result in occurrence of the respective diseases. Though methods are available for the detection of individual pathogenic organisms, it is neither practicable nor necessary to adopt such tests in routine examination of water supplies. When pathogenic organisms are present, they are almost always far outnumbered by other faecal organisms and the latter are easier to detect. If they are not found, it can be taken that disease producing organisms too are absent.

The organisms most commonly used as indicators of pollution are *E. coli* and the coliform group as a whole. *E. coli* is of undoubted faecal origin, but as to the exact significance of the other members of the coliform group there is difference in opinion. From the practical point of view, their presence should be taken as indicative of faecal pollution unless their nonfaecal origin can be proved.

Faecal streptococci, regularly present in faeces in varying numbers, are usually fewer than *E. coli*, and probably die and disappear at the same rate. But usually they die more rapidly than other members of the coliform group. Therefore the finding of faecal streptococci and coliforms but not *E. coli* is also confirmatory of faecal pollution.

Anaerobic spore bearing organisms of which the most characteristic is *Cl. welchii* are also regularly present in the faeces, though in much smaller numbers than *E. coli*. Their spores can survive much longer than organisms of the coliform group in water and usually resist chlorination at the doses used in water works practice. The pre-

sence of *Cl. welchii* in natural water suggests that faecal contamination has occurred and in the absence of organisms of the coliform group, indicates that such contamination occurred some considerable time ago.

Standards: Water in the distribution system, whether treated or not, should not contain any organisms indicative of faecal origin. The presence of organisms of the coliform group is indicative of faecal pollution unless a nonfaecal origin can be proved. The coliform group includes all Gram negative nonspore forming rods capable of fermenting lactose with the production of acid and gas at 37°C in less than 48 hours.

E. coli is definitely of faecal origin and is defined as a Gram negative nonspore forming rod capable of fermenting lactose with the production of acid and gas both at 37°C and 44°C in less that 48 hours. It produces indole in peptone water containing tryptophan and is incapable of utilising sodium citrate as its sole source of carbon.

Piped supplies: 1) Water entering the distribution system: a) Disinfected supplies: It should be free from any coliform and no coliform should be demonstrable in any sample of 100 ml. b) Nondisinfected supplies: No *E. coli* should be present in any sample of 100 ml. In its absence, upto three coliform organisms per 100 ml may be tolerated in occasional samples. If repeated samples show coliforms, steps should be taken to find out the source and remove the sources of pollution. If the number of coliforms increases to more than three per 100 ml the supply should be considered unsuitable, without disinfection.

2) Water in the distribution system: Water may be of excellent quality when it enters the system but coliforms may gain access to it in the distribution system from booster pumps, from the packing used in the joining of mains, or from washers on service taps. In addition, contamination can occur from outside through cross connections, back siphonage, defective service reservoirs and water tanks, damaged or defective hydrants or washouts or through inexpert repairs to domestic plumbing systems. The following standards are recommended:

1. Throughout any year, 95 per cent of samples should not contain any coliform organisms in 100 ml.
2. No samples should contain *E. coli* in 100 ml.
3. No samples should contain more than 10 coliform organisms per 100 ml.
4. Coliform organisms should not be detectable in 100 ml of any two *consecutive* samples.

Individual or small community supplies. The coliform count should be less than 10 per 100 ml. Persistent failure to achieve this by relatively simple sanitary measures, particularly if *E. coli* is repeatedly found, should lead to condemnation of the supply.

Methods

1. *Total content of microorganisms*: Plate count: A determination of the total number of viable bacteria in a water sample, although of limited value by itself, gives an indication of the amount and type of organic matter present in the supply. The test is carried out in duplicate at 20–22°C and 37°C. Those that grow at 37°are those most likely to be associated with organic material of human or animal origin, whereas those growing at a lower temperature are mainly saprophytes that normally inhabit the water or are derived from soil and vegetables.

The agar count at 22°C gives an indication of the amount of decomposing organic matter in the water available for bacterial nutrition. Though most bacteria growing at 22°C are nonpathogenic to humans on general grounds, the greater the amount of organic matter present, the more likely is the water to be contaminated with parasitic and potentially pathogenic organisms. The agar count at 37°C is a more important index of dangerous pollution. A high count is undesirable. They may cause spoilage of food and drink in the home or more so in the factory. 37°C agar count is of particular value in the control of filtration and chlorination. With slow sand filters the count

should show a 95–98 per cent reduction on that of raw water. A rise in colony count is the usual signal of some defect in the filter beds demanding immediate attention.

2. *Detection of coliform organisms* and *E. coli*.
a) Presumptive coliform count — Multiple tube technique: The test is called presumptive because the reaction observed may occasionally be due to the presence of some other organisms or combination of organisms and the presumption that the reaction is due to coliform organisms has to be confirmed.

An estimate of the number of coliform organisms is usually made by adding varying quantities of water (0.1 ml–50 ml) to bile salt lactose peptone water (with an indicator for acidity) and incubating at appropriate temperatures. Acid and gas formation indicate growth of coliform bacilli. Thus it is possible to state the smallest quantity of water containing a coliform bacillus and to express the degree of contamination with this group of organisms.

The following range is put up:

One 50 ml quantity of water to 50 ml double strength medium.
Five, 10 ml quantiaties each to 10 ml double strength medium.
Five, 1.0 ml quantities each to 5.0 ml single strength medium.
Five, 0.1 ml quantities each to 5.0 ml single strength medium.

MacConkey's fluid medium (modified) is used. The range of quantities depends on the likely strength of contamination. For highly contaminated waters, smaller volumes are tested. The bottles are incubated at 37°C and examined after 18–24 hours. The 'presumptive positives' are read off and remaining negative bottles are reincubated for another 24 hours. Any further positives are added to the previous figures. The probable number of coliforms per 100 ml are read off from the probability tables of McCrady.

b) Differential coliform test: The Eijkman test is usually employed to find out whether the coliform bacilli detected in the presumptive test are *E. coli*. After the usual presumptive test, subcultures are made from all the bottles showing acid and gas to fresh tubes of single strength MacConkey's medium already warmed to 37°C. They are incubated at 44°C and examined after 24 hours. Incubation at 44°C should be carried out in thermostatically controlled water baths that do not deviate more than 0.5°C from 44°C. Those showing gas in Durham's tubes contain *E. coli*. From the number of positive tubes obtained, results are read off the probability tables. When using MacConkey's broth for this test, it is recommended that the presumptive positive tubes derived from chlorinated waters should be plated on to a solid medium to confirm the presence of coliforms, since false reactions in MacConkey's broth, both at 37°C and 44°C, may be caused by spore bearing anaerobic organisms. Further confirmation of the presence of *E. coli* can be obtained by testing for indole production and citrate utilisation.

c) Membrane filtration method: A measured volume of water is filtered through millipore fil-

TABLE 64.3
Classification of drinking water according to bacteriological tests

	Presumptive coliform count per 100 ml	*E. coli* count per 100 ml
Class I Excellent	0	0
Class II Satisfactory	1-3	0
Class III Suspicious	4-10	0
Class IV Unsatisfactory	More than 10	0,1 or more

ter. All the bacteria present are retained on its surface. It is placed on suitable media face upwards and incubated at the appropriate temperature and the colonies that develop on the surface of the membrane are counted. After 18 hours of incubation the presumptive coliform counts and *E. coli* counts can be directly made.

The multiple tube and membrane techniques do not give strictly comparable results.

(All details regarding techniques when using millipore membranes are described in Application Manual 'AM302' of Millipore Corporation, Bedford, Massachusetts, USA.)

3. Detection of faecal streptococci: Subcultures are made from all positive bottles in the presumptive coliform test into tubes containing 5.0 ml of glucose azide broth (Hannay and Norton, 1947). The presence of *Str. faecalis* is indicated by the production of acid in the medium within 18 hours at 45°C. Positive tubes should be plated on to MacConkey's agar for confirmation.

Millipore membrane technique can also be adopted for this purpose.

Since bile salt in MacConkey's medium shows variation in its inhibitory power other media have been devised. Bile salt is replaced by 1% Teepol 610 or by 0.1% sodium lauryl sulphate. The lactose formate glutamate medium of Folpmer gives better results, is cheaper and constant in its composition.

4. Examination for Cl welchii: This is tested by incubating varying quantities of the water in question in litmus milk medium (anaerobically) at 37°C for five days and looking for stormy fermentation.

Virological examination

The possibility of the transmission of virus disease through water free of coliforms cannot be overruled though positive evidence is lacking. None of the generally accepted methods of sewage treatment yields virus free effluents. Enteroviruses, echoviruses, parvoviruses, reoviruses and adenoviruses have been found in water, the first named being most resistant to chlorination. If a treated water is free of enteroviruses it can be considered safe though one is not certain whether infective hepatitis viruses also would have been simultaneously destroyed.

In practice, 0.5 mg/litre of free chlorine for one hour is sufficient to inactivate virus, even in water which was originally polluted. If not even one plaque forming unit (PPU) of virus can be found in one litre of water, it can be reasonably assumed that it is safe to drink.

It is not practicable to test for presence of viruses as frequently as bacteriological examination. The frequency of examination done should be based on local circumstances.

The same procedure for collecting samples as in the case of bacteriological examination should be adopted. The sample should be at least two litres in volume and it should be sent to the laboratory with the least delay.

Biological examination

This is of value in determining the causes of objectionable tastes and odours in water, controlling remedial treatments, in interpretation of the results of chemical analyses, and in understanding the causes of clogging in distribution pipes and filters.

Some of the animalcules which are more or less firmly attached to the inside of the mains, though nonpathogenic themselves, may harbour pathogenic organisms or viruses thus protecting them from destruction by chlorine. Chlorination at the doses normally employed is ineffective against amoebic cysts. The following methods can be used to collect samples from piped supplies:

1. From taps: A large volume of water is filtered through membrane filters. After drying they are treated with immersion oil, which renders them transparent. Direct microscopy is then done. Alternatively a special filter device can be attached to the tap and a large volume of water is allowed to pass through it and the deposit examined.

2. From mains: A special nylon net or cotton bag is attached to the outlet of a hydrant and a section of the main washed through using a high water flow rate. One can also 'swab' a section of the mains by special devices. The debris obtained is examined.

In sampling water from nonpiped supplies, the procedure is the same as for bacteriological examination. Clean bottles of two litre capacity can be used instead of sterilised bottles. The temperature of the sample should be maintained at its original value and despatched speedily to the laboratory.

Examination for specific pathogens

In special situations, specific pathogens such as *S. typhi* and *V. cholerae* may have to be looked for in drinking water. They may be isolated by employing enrichment and selective media. For detection of *S. typhi* double strength selenite medium with an equal volume of water added is incubated and subcultured in Wilson and Blair medium. For isolation of *V. cholerae* alkaline peptone water (10 X) mixed with nine times its volume of water is incubated and subcultured on bile salt agar. The isolation of pathogenic bacteria may also be done by the membrane filter method already described, but using selective media for culture.

BACTERIOLOGY OF MILK

Milk always contains bacteria of different types derived from various sources such as the animal from which the milk is obtained, the milker, the milking equipment and in poor countries, the water used for adulteration. The dust from the environment may also contribute.

Bacteria are derived from the milk ducts of the udder even when utmost aseptic precautions are taken. The numbers vary from quarter to quarter and from animal to animal. They are highest in the foremilk and lowest in the strippings. The average plate count in aseptically drawn milk has been variously estimated from ten per ml to several thousand per ml. Probably if drawn from healthy cows, taking all aseptic precautions it would be less than 100 per ml. Unclean udders and dust in milking sheds also contribute to bacteria in milk. Unsterile milking equipment is a major source of contamination. Disease in the animal such as mastitis, tuberculosis and brucellosis is a major hazard. An easily avoidable but none the less dangerous source is from the human personnel involved in the milking. Though the number of organisms are few, they are of considerable public health importance, if the milker happens to be a carrier of typhoid, paratyphoid, dysentery and food poisoning bacilli, haemolytic streptococci or *Corynebacterium diphtheriae*.

Type of bacteria in milk

These can be classified as below:

1. Acid forming bacteria: Most of these gain access to the milk from contaminated utensils. Many of them are probably of vegetable origio. Some may be from the udder such as staphylococci. The commonest organisms are lactic streptococci including *Str. lactis* and *Str. faecalis*. Lactobacilli are also found. These organisms ferment the lactose in the milk producing acids, mainly lactic acids, which leads to the formation of a smooth gelatinous curd.

2. Alkali forming bacteria: These consist of *Alkaligenes spp*, some aerobic spore bearers and some *Achromobacter*. These render the milk alkaline.

3. Gas forming bacteria: Coliform bacilli are the commonest. Others are *Cl. welchii* and *Cl. butyricum*. Acid aod gas are produced. A smooth gelatinous curd riddled with gas bubbles is formed. Coliform bacilli are responsible for the ropiness in milk.

4. Proteolytic bacteria: Spore bearing aerobes such as *B. subtilis* and *B. cereus*, *Proteus vulgaris*, staphylococci and micrococci come under this heading.

5. *Inert bacteria:* Bacteria which produce no visible change in milk are called *inert*. These include some cocci of the udder, members of the *Achromobacter* group and most of the pathogenic organisms in the milk.

6. *Human milk:* Before the infant is fed, breast milk contains in small numbers, *Staph. epidermidis* in every sample, *Str. mitis* in 69 per cent of samples, *Gaffkya tetragena* in 19 per cent and *Staph. aureus* in 13 per cent. A few other species may be found in under 10 per cent of samples. After feeding, the same organisms are seen but in larger numbers, most of them probably derived from the infant's mouth and maternal skin.

Milk borne diseases

1. *Infections of animals that can be transmitted to man:* The most important diseases are tuberculosis, brucellosis, streptococcal and staphylococcal infections, salmonellosis and Q fever. Diseases of less importance include cowpox, vaccinia and pseudocowpox (milker's nodes) which are usually transmitted to milkers in the act of milking rather than through ingestion of milk. Foot and mouth disease, anthrax and leptospirosis have been transmitted on rare occasions. Diseases such as tuberculosis and brucellosis can lead directly or indirectly to contamination of milk. The tick borne encephalitis virus may be transmitted through goat milk. It has been suggested that other viruses such as poliovirus and other enteroviruses may be transmitted through milk but the evidence is not convincing. Milk borne infectious hepatitis has been reported on several occasions.

Occasionally milk may be contaminated with *Streptobacillus moniliformis* from the nasal secretion of rats and with *Campylobacter fetus*, subsp. *jejuni* from animal faeces. *Yersinia enterocolitica* is not uncommon in milk and may give rise to gastroenteritis if present in large numbers. *Toxoplasma gondii* is sometimes found in human milk.

The organisms that cause all the diseases mentioned above are destroyed by adequate pasteurisation

2. *Infections primary to man that can be transmitted through milk:* a) Enteric infections: These are caused by the consumption of milk which has been contaminated with water contaminated by human excreta. A less common source are the human carriers of enteric infections employed in the dairies. The diseases are typhoid and paratyphoid fevers, shigellosis, cholera (rarely) and diarrhoea due to enteropathogenic *E. coli*. b) Streptococcal infections: Cows may have udder or teat infections and the organisms get into the milk. Milk handlers may be carriers and may contaminate the milk. c) Staphylococcal food poisoning: Milk from cows suffering from staphylococcal mastitis is contaminated with the organism. If the milk is consumed after being allowed to remain at temperatures favourable to its multiplication the enterotoxin produced by the organism causes food poisoning. Many such outbreaks have been reported. d) Diphtheria: Milk contaminated either from a human carrier or more usually through diphtheritic lesions on the teats caused by the practice of wet milking, when consumed unpasteurised, leads to disease. e) Tuberculosis: Milk contaminated by excretions from humans suffering from tuberculosis, when consumed, leads to the disease.

Bacteriological examination

Bacteriological examination of milk attempts to determine the number and type of bacteria present in milk as well as to verify whether the methods of sterilisation employed have been effective. Milk has been classified into several grades of purity (tuberculin tested, certified, pasteurised, sterilised, etc.) and different standards have been laid down for each of these grades.

The routine bacteriological examination of milk consists of the following.

1. *Viable count:* This is estimated by doing plate counts with serial dilutions of the milk sample. Raw milk always contains bacteria varying in number from about 500 to several million per ml.

2. *Test for coliform bacilli:* This is tested by inoculating varying dilutions of milk into MacConkey's fluid medium and noting the production of acid and gas after incubation. Contamination with coliforms comes mainly from dust, dirty utensils and dairy workers.

3. *Methylene blue reduction test:* This is a simple substitute for the viable count. It depends on the reduction of methylene blue by bacteria in milk when incubated at 37°C in complete darkness. The rate of reduction is related to the degree of bacterial contamination. Raw milk is considered satisfactory, if it fails to reduce the dye in 30 minutes under standard conditions.

The Resazurin test is similar, but the dye resazurin, on reduction, passes through a series of colour changes — from blue to pink to colourless — the shade of colour after incubation with milk for a particular period of time depending on the degree of contamination. Generally the 10-minute resazurin test is done, in which the shade of colour is noted after incubation with the milk for ten minutes.

4. *Phosphatase test:* This is a check on the pasteurisation of milk. The enzyme phosphatase normally present in milk is inactivated if pasteurisation has been carried out properly. Residual phosphatase activity indicates that pasteurisation has not been adequate.

5. *Turbidity test:* This is a check on the 'sterilisation' of milk. If milk has been boiled or heated to the temperature prescribed for 'sterilisation', all heat coagulable proteins are precipitated. If ammonium sulphate is then added to the milk, filtered and boiled for five minutes, no turbidity results. This test can distinguish between pasteurised and 'sterilised' milk.

6. *Examination for specific pathogens:* a) Tubercle bacillus: The milk is centrifuged at 3000 r.p.m. for 30 minutes and the sediment inoculated into two guinea pigs. The animals are observed for a period of three months for tuberculosis. Tubercle bacilli may also be isolated in culture. Microscopic examination for tubercle bacilli is unsatisfactory. b) Brucella: Isolation of brucella may be attempted by inoculating cream heavily on serum dextrose agar or by injecting centrifuged deposit of the milk sample intramuscularly into guinea pigs. The animals are sacrificed after six weeks and the serum tested for agglutinins and the spleen inoculated in culture media.

Brucellosis in animals can be detected also by demonstrating the antibodies in milk, by the milk-ring or the whey-agglutination tests.

In conclusion, the tests adopted for the routine examination of milk should reveal the degree of bacterial contamination and thereby enable us to know whether the milk is produced and handled in a hygienically satisfactory manner. The plate count gives a rough and direct assessment of the viable bacteria in the milk. It is easily explainable to the producer and gives a fair idea of the improvement or deterioration in the conditions of production. The coliform test is useful not only in warning us of possible faecal contamination, but also gives us an indication of contamination from dust or unsterile utensils. Phosphatase test if positive after proper pasteurisation of milk, shows contamination after pasteurisation. The dye test is a rough and quick test as to the satisfactoriness of the milk as its arrives from the producer.

All these tests have their own drawbacks. Each has value if the limitations of the test are kept in mind and the test is properly performed. For the details of the various tests and the minutiae of their interpretaion, standard monographs have to be consulted.

BACTERIOLOGY OF AIR

In the course of a day a man respires about *500 c. ft.* of air. Hence the bacterial content of the air he breathes is important, particularly so when it contains pathogens, and that too in significant numbers likely to cause disease. The bacterial

content of air depends on the location, i.e., whether it is outdoor air or indoor air.

The bacterial content of outdoor air depends on many factors such as the density of human and animal population, the nature of the soil, the amount of vegetation, the atmospheric conditions such as humidity, temperature and wind conditions, rainfall, and sunlight. Most of the bacteria are nonpathogenic and even the rare pathogen that may contaminate the air is rarely able to survive the adverse conditions of the outdoor air to cause disease.

Spores and fragments of moulds are very much more than bacteria. Bacteria in upper air consist largely of aerobic spore bearing bacilli and to a much less extent organisms such as *Achromobacter*, *Sarcina* and *Micrococci*. The latter are mainly derived from soil and surface dust and are likely to be carried for long distances horizontally and vertically for miles. Infective materials are seldom carried for more than short distances and their capacity to cause infections is impaired, except in rare instances such as foot and mouth disease virus. Pathogenic bacteria do not multiply in air.

In the case of indoor air, the bacteria may be distributed through gross droplets and droplet nuclei from nose and mouth and through dust particles. Dust consists of particles of varying size originating from animal, vegetable or mineral sources. The ultimate source of common pathogenic organisms is dust derived from humans. Nasal secretions via ala nasi and upper lip get carried by hands to skin, clothing and bedding from where they get detached as dust. Organisms may also get directly detached from the skin of different parts of the body including perineum and septic wounds. Intestinal organisms through dried particles of faeces from napkins, and infants also get disseminated. The heavy particles fall to the ground while those 1μ or less in diameter remain mostly suspended in air. Haemolytic streptococci from cases or carriers, tubercle bacilli and diphtheria bacilli and staphylococci are found in ward dust where such cases are treated. Under favourable conditions they may remain alive for many weeks. Bed clothes are an abundant source of bacteria-laden dust. Desquamated epithelial cells from the body get liberated into the environment through physical activity. The stream of air enveloping the body also serves as a source of organisms in dust.

During coughing, sneezing and talking, varying numbers of droplets are expelled from the body, varying in size from less than one to 15 μm. Depending on their size they travel or remain suspended in air or fall to the ground and in the process get evaporated, the smaller the size, the faster. On evaporation they get converted to very minute particles called 'droplet nuclei' and their fate depends on air currents in the atmosphere. The viability of bacteria in the droplet nuclei depends on numerous factors and is unpredictable. Experiments show that the proportion of dust particles and droplet nuclei reaching the lung depend on their size. All particles over 5 μm are retained in the nose, most of 1μm reach the lung and are retained in the alveoli but below 1μm the proportion retained in the lung diminishes

Infective or potentially infective droplets may also be liberated in the form of aerosols by various laboratory procedures, dental manipulations and in the flushing of water closets.

Measurement of air contamination

Sedimentation method: Open plates of culture media are exposed for specific periods, e.g., half to one hour, the plates are incubated at 37°C for 24 hours and the number of colonies counted. When pathogenic staphylococci and streptococci are looked for, blood agar plates are used. This method gives us an idea of the relative numbers and species of microorganisms present in air and is specially used for testing air in surgical theatres and hospital wards.

Slit sampler Since the plate exposure method has many limitations, a more elaborate method, the slit sampler, has been introduced. In this, a known volume of air is directed onto a plate through a slit 0.25 mm wide, the plate being mechanically rotated so that the organisms are

evenly distributed over it. One cubic foot of air per minute is allowed to pass through the slit and samples of 1–10 cubic feet or more are tested.

Though there are no accepted standards of bacterial pollution of air, it has been suggested that the levels of infection that are acceptable for factories, homes, offices and such places as 50 per ft^3 and for surgical operating theatres providing for most forms of surgery as 10 per ft^3 and in theatres where operations on the central nervous system or dressings for burns are done as about one per ft^3. The great majority of bacteria found in the air are harmless saprophytes or commensals, and even in hospital wards not more than one per cent and commonly 0.01 – 0.1 per cent of air borne bacteria are pathogens.

Bacteriological examination of environmental dust

Sweep plate: This is used to examine personal clothing, bed clothes and domestic fabrics such as curtains, carpets, etc. for the presence of pathogenic bacteria liable to be liberated in dust. A Petri dish containing the medium is removed from its lid and rubbed to and fro on the surface of the fabric, the medium facing the fabric. The edges of the plate while scraping the fabric throw up the dust onto the medium. About 10 or more sweeps are made with the plate.

Dust on floors: Cottonwool moistened with broth are used to collect the sample and the swab is plated out.

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65 Medical Mycology

Fungi had been recognised as causative agents of human disease earlier than bacteria. Fungi causing favus (*Trichophyton schonleinii*) and thrush (*Candida albicans*) had been described as early as in 1839. In spite of the earlier beginnings, the study of pathogenic fungi has received only scant attention in comparison with the study of the other pathogens. This is probably due to the relatively benign nature of the common mycotic diseases and because the techniques employed in mycology are more those of botanists than of bacteriologists. Fungus infections, however, are extremely common and some of them are serious and even fatal. With the control of most bacterial infections in the developed countries, fungus infections have assumed greater importance. For instance, it has been stated that in the USA fungus infections cause as many fatalities today as whooping cough, diphtheria, scarlet fever, typhoid, dysentery and malaria put together. Most fungi are soil saprophytes, and human infections are mainly opportunistic. Modern advances in treatment, such as antibiotics, steroids and immunosuppressive agents have led to an increase in opportunistic fungus infections.

Fungi are eukaryotic protists that differ from bacteria and other prokaryotes in many ways. They possess rigid cell walls containing chitin, mannan and other polysaccharides. The cytoplasmic membrane contains sterols. They possess true nuclei with nuclear membrane and paired chromosomes. They divide asexually, sexually or by both processes. They may be unicellular or multicellular. The cells show various degrees of specialisation.

The simplest type of fungus is the unicellular budding yeast. Elongation of the cell produces a tubular, thread-like structure called *hypha*. A tangled mass of hyphae constitutes the *mycelium*. Fungi which form mycelia are called moulds or filamentous fungi. Hyphae may be septate or nonseptate. The septa, when present, have holes through which free flow of cytoplasmic material can take place. In a growing colony of filamentous fungus, the mycelium can be divided into the vegetative mycelium which grows into the medium and the aerial mycelium which projects from the surface.

Depending on cell morphology, fungi can be divided into four classes: yeasts, yeast-like fungi, moulds and dimorphic fungi.

Yeasts are unicellular fungi which occur as spherical or ellipsoidal cells and reproduce by simple budding. On culture, they form smooth, creamy colonies. The only pathogenic yeast is *Cryptococcus neoformans*.

Yeast-like fungi grow partly as yeast and partly as elongated cells resembling hyphae. The latter form a *pseudomycelium*. *Candida albicans* is a pathogenic yeast-like fungus.

Moulds or filamentous fungi form true mycelia and reproduce by the formation of different types of spores. Dermatophytes are examples of pathogenic moulds.

Dimorphic fungi can occur as filaments or as yeasts depending on the conditions of growth. In host tissues or cultures at 37°C they occur as yeasts, while in the soil and in cultures at 22°C

they appear as moulds. Most fungi causing systemic infections are dimorphic fungi.

The systematic classification of fungi, based on their sexual spore formation, recognises four classes. *Phycomycetes* are lower fungi which have nonseptate hyphae and form endogenous asexual spores, called 'sporangiospores', contained within swollen sac-like structures called 'sporangia'. Phycomycetes also produce sexual spores known as *oospores* in some fungi and *zygospores* in some others. The other three classes (the higher fungi) have septate hyphae and form exogenous asexual spores called 'conidia'. The *Ascomycetes* form sexual spores *(ascospores)* within a sac or ascus. Ascomycetes include both yeasts and filamentous fungi. The *Basidiomycetes* form sexual spores *(basidiospores)* on a 'basidium' or base. The fourth class, *Fungi imperfecti* (also called Deuteromycetes or Hyphomycetes), is a provisional group consisting of fungi whose sexual phases have not been identified. Most fungi of medical importance belong to this group.

The laboratory diagnosis of fungus infections is made by microscopic examination of materials from the lesions and by morphological study of fungus isolates. Tissue specimens, such as skin scrapings are generally examined as wet mounts after treatment with 10% potassium hydroxide. The alkali digests cells and other tissue materials, enabling the fungus elements to be seen clearly. Small bits of fungus colonies may be teased on to a slide and mounted in lactophenol cotton blue for microscopic study. Slide culture provides a useful technique for the study of fungus morphology. The periodic acid Schiff (PAS) and methanamine silver stains are valuable methods for the demonstration of fungal elements in tissue sections.

The commonest culture media used in mycology are Sabouraud's glucose agar (pH 5.4), Czapek-Dox medium and Cornmeal agar. The addition of antibiotics prevents bacterial contamination. Cycloheximide (actidione) incorporated in the medium inhibits many contaminant moulds. Cultures are routinely incubated in parallel at room temperature (22°C) for weeks and at 37°C for days. Identification is based on the morphology of the fungus and of its colony. Biochemical and serological tests, which form the mainstay of bacterial identification, are seldom employed in mycology. Diagnostic mycology rests largely on a detailed study of the morphological evolution of the isolate and has therefore been termed an 'exercise in contemplative observation'.

Growth characters useful for identification are the rapidity of growth, colour and morphology of the colony on the obverse and pigmentation on the reverse. The morphology of hyphae, spores and other structures is studied in teased mounts or slide cultures. Hyphal diameter, presence or absence of septa and of special structures are of diagnostic importance. Special hyphal structures frequently found are spring-like helical coils (spiral hyphae), localised swellings formed by tightly twisted hyphae resembling tennis racquets (racquet hyphae) and numerous short branches appearing at the ends of hyphae (favic chandelier) (Fig. 65.1). The morphology of asexual spores or conidia is of diagnostic importance. They may be small, single-celled 'microconidia' or large, single or multicelled 'macroconidia'. The type of spore formation is distinctive for different fungi. 'Blastospores' are formed by budding, as in yeasts. 'Arthrospores' are formed along the mycelium by segmentation and condensation of hyphae. 'Chlamydospores' are thick walled resting spores formed by rounding up and thickening of hyphal segments.

MYCOSES
(FUNGUS INFECTIONS)

Human fungus infections are broadly of two types — superficial and deep seated (systemic). Superficial infections are by far commoner and comprise the various types of tinea or ringworm affecting the skin, hair and nails. These are mild though chronic diseases. Fungi causing superficial mycoses are specialised saprophytes, with the capacity to digest keratin. Systemic mycoses

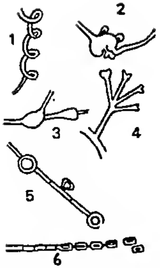

Fig. 65.1 Mycelial forms and asexual spores of fungi 1 spiral hypha, 2 nodular organ, 3. racquet mycelium, 4 favic chandelier, 5 chlamydospores along hypha, 6 arthrospores

are caused by fungi that are mostly soil saprophytes. Infection is accidental. Systemic mycoses occur in varying degress of severity, ranging from asymptomatic infection to fatal disease.

A third type of fungus infection is opportunistic infection, occurring in patients with debilitating diseases such as cancer or diabetes, or in whom the physiological state has been upset by immunosuppressive drugs, steroids, X-rays or broad spectrum antibiotics. Opportunistic infections are caused mainly by fungi that are normally avirulent, such as *Mucor*, *Penicillium* and *Aspergillus*.

SUPERFICIAL MYCOSES

Superficial mycoses are of two types — surface infections and cutaneous infections. In the former, the fungi live exclusively on the dead layers of the skin and its appendages. They have no contact with living tissue and hence elicit no inflammatory response. The only changes produced are cosmetic effects. *Tinea. versicolor*, *Tinea nigra* and *Piedra* fall into this group. The most important cutaneous infection is dermatophytosis caused by a group of related fungi called the *dermatophytes*. Infection is generally confined to the cornified layer of the skin and its appendages, but a variety of inflammatory and allergic responses are induced in the host by the presence of the fungi and their metabolic products. Another type of cutaneous infection is caused by *Candida albicans*. Though Candida infection is mostly confined to the skin and mucosa, it can also cause systemic disease rarely, involving any organ. Candida infection, therefore, represents a bridge connecting superficial and deep mycoses.

Pityriasis versicolor

Pityriasis versicolor (Tinea versicolor) is a chronic, usually asymptomatic involvement of the stratum corneum, characterised by discrete or confluent macular areas of discolouration or depigmentation of the skin. The areas involved are mainly the chest, abdomen, upper limbs and back. The causative agent is a lipophilic, yeast-like fungus *Pityrosporum orbiculare (Malassezia furfur)*. The disease is worldwide in distribution, but is particularly prevalent in the tropics. It occurs mainly in young adults. Diagnosis is established by examination of skin scrapings, which show an abundance of yeast-like cells and short, branched filaments (Fig. 65.2). The fungus can be grown on Sabouraud's agar, covered with a layer of olive oil. The fungus may by demonstrated on the normal skin also and the disease may be considered an opportunisitic infection.

Tinea nigra

Tinea nigra is a localised infection of the stratum corneum, particularly of the palms, producing black or brownish macular lesions. It is found in the tropics and is caused by *Cladosporium mansoni* in Asia and Africa and by *C. werneckii* in America. Skin scrapings show brownish, branched, septate hyphae and budding cells. Colonies on Sabouraud's medium are grey or black in colour.

Fig. 65.2 *Pityrosporum orbiculare*: in stratum corneum. Clusters of small, round, budding yeast cells are interspersed among septate branching hyphae. Skin scraping, KOH preparations.

Piedra

Piedra is a fungus infection of the hair, characterised by the appearance of firm, irregular nodules along the hair shaft. The nodules are composed of fungus elements cemented together on the hair. Two varieties of piedra are recognised — black piedra caused by *Piedraia hortai* and white piedra caused by *Trichosporon cutaneum*.

Dermatophytoses

The dermatophytes are a group of closely related filamentous fungi that infect only superficial keratinised tissues — the skin, *hair and nails*. They cause a variety of clinical conditions, collectively known as dermatophytoses, popularly called tinea or ringworm. The term dermatomycosis, sometimes used as a synonym, would include also skin lesions produced by other fungi such as *Candida albicans* and also the cutaneous manifestations of systemic mycoses.

Dermatophytes have been classified into three genera — Trichophyton, Microsporum and Epidermophyton. About 40 species of dermatophytes are known to cause infection in man and animals.

In lesions, dermatophytes appear as hyphae and arthrospores. In cultures on Sabouraud's agar, they form characteristic colonies consisting of septate hyphae and two types of asexual spores, microconidia and macroconidia. Sexual spores of some species have been identified recently. Differentiation into the three genera is based mainly on the nature of macroconidia (Fig 65.3).

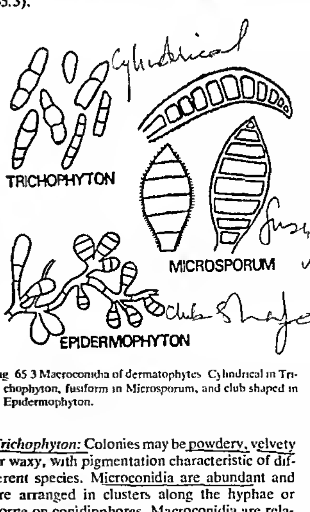

Fig. 65.3 Macroconidia of dermatophytes. Cylindrical in Trichophyton, fusiform in Microsporum, and club shaped in Epidermophyton.

Trichophyton: Colonies may be powdery, velvety or waxy, with pigmentation characteristic of different species. Microconidia are abundant and are arranged in clusters along the hyphae or borne on conidiophores. Macroconidia are rela-

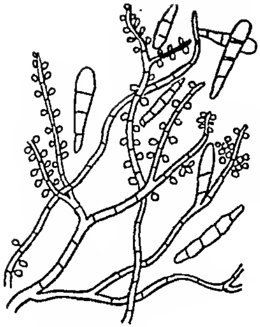

Fig. 65.4 *Trichophyton rubrum* culture mount showing microconidia along sides of hyphae, and long cylindrical macroconidia.

tively scanty. They are generally elongated, with blunt ends (Fig. 65.4). Macroconidia have distinctive shapes in different species and are of importance in species identification. Some species possess special hyphal characters, such as spiral hyphae, racquet mycelium and favic chandeliers. Trichophytons infect skin, hair and nails. *T. rubrum* is the most common species infecting man. It often causes chronic, treatment resistant lesions.

Microsporum: Colonies are cottony, velvety or powdery, with white to brown pigmentation. Microconidia are relatively scanty and are not distinctive. Macroconidia are the predominant spore form. They are large, multicellular, spindle shaped structures, borne singly on the ends of hyphae. Microsporum species infect the hair and skin, but usually not the nails.

Epidermophyton: Colonies are powdery and greenish yellow in colour. Microconidia are absent. Macroconidia are multicellular, pear shaped and typically arranged clusters. Epider-

TABLE 65.1
Some characteristic of common dermatophytes

Species	Colony	Morphology
T. rubrum	Velvety, red pigment on reverse	Few, long, pencil shaped macroconidia
T. mentagrophytes	White to tan, cottony or powdery. Pigment variable	Clusters of microconidia. Cigar shaped macroconidia with terminal rat-tail filaments
T tonsurans	Cream or yellow, with central furrows	Abundant microconidia. Thick walled, irregular macroconidia
T schoenleinii	Smooth, waxy, brownish	Hyphal swellings, chlamydospores, favic chandelier
T violaceum	Very slow growing. Waxy, violet to purple pigment	Distorted hyphae, Conidia rare
M. audouinii	Velvety, brownish, slow growing	Thick walled chlamydospores, conidia rare and irregular
M canis	Cottony, orange pigment on reverse	Abundant, thick walled spindle shaped macroconidia with upto 15 septa
M gypseum	Powdery, buff-coloured	Abundant, thin walled macroconidia with 4-6 septa
E. floccosum	Yellowish green. Powdery	Club shaped macroconidia in clusters

mophyton attacks the skin and nails, but not the hair. The genus contains only one species, *E. floccosum.*

Table 65.1 shows the important differentiating features of some common dermatophytes.

Pathogenicity: Dermatophytes grow only on the keratinised layers of the skin and its appendages and do not ordinarily penetrate into the living tissues. The mechanisms of pathogenesis in dermatophytosis are not clear. Fungal products may be responsible for inciting local inflammation. Hypersensitivity to fungus antigens may play a role in pathogenesis and is probably responsible for the sterile vesicular lesions sometimes seen in sites distant from the ringworm. These lesions are called 'dermatophytids' (or the 'id' reaction). Hypersensitivity can be demonstrated by skin testing with the fungus antigen, trichophytin.

Clinically, ringworm can be classified depending on the site involved. *Tinea corporis (Tinea glabrosa)* is ringworm of the smooth or non-hairy skin of the body. A special type is *Tinea imbricata* which is found in the tropics and is characterised by extensive concentric rings of papulosquamous scaly patches. *Tinea cruris* is involvement of the groin and the perineum, *Tinea barbae* or barber's itch is involvement of the bearded areas of the face and neck. *Tinea ~~pedis~~* or athletes' foot is ringworm of the foot and *Tinea capitis* ringworm of the scalp. 'Favus' is a chronic type of ringworm in which dense crust (scutula) develop in the hair follicles, which lead to alopecia and scarring. Scalp infection sometimes produces severe boggy lesions with marked ioflammatory reaction called 'kerion'. Table 65.2 lists the clinical types of dermatophytoses and their common causative agents.

Laboratory diagnosis: The routine method of diagnosis is by the examination of KOH mounts. Scrapings are taken from the edges of ringworm lesions. The specimen is mixed with a drop of 10% KOH on a slide, and after placing a coverslip, the preparation is gently heated to bring about 'clearing'. Microscopy reveals branched septate hyphae (Fig. 65.5). Selection of infected hair for examination is facilitated by exposure to UV light (Wood's lamp). Infected hair will be fluorescent. Two types of hair infection may be distinguished in wet mounts, 'ectothrix' in which arthrospores are seen as a sheath surrounding the hair and 'endorthrix' in which the spores are inside the hair shaft (Fig. 65.6). Demonstration of fungus in nails may be difficult and may be possible only after clearing with KOH for a day or two.

Species identification is possible only by cultural examination. Specimens are inoculated on Sabouraud's medium (with antibiotics and cycloheximide) and incubated at room temperature.

TABLE 65 2

Clinical types of dermatophytoses and their common causative agents

Disease	Common causative agents
Tinea capitis	*Microsporum* any species, *Trichophyton* most species
Favus	*T. schoenleinii, T. violaceum, M. gypseum*
Tinea barbae	*T. rubrum, T. mentagrophytes, T. verrucosum*
Tinea imbricata	*T. concentricum*
Tinea corporis	*T. rubrum* and any other dermatophyte
T. cruris	*E. floccosum, T. rubrum*
T. pedis	*T. rubrum, E. floccosum*
Ectothrix hair infection	*Microsporum* species, *T. rubrum, T. mentagrophytes*
Endothrix hair infection	*T. schoenleinii, T. tonsurans, T. violaceum*

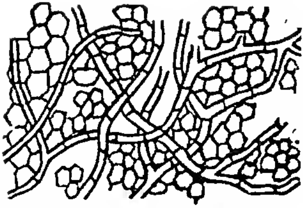

Fig. 65.5 Dermatophyte hyphae in skin scraping, KOH mount. Partly digested epithelial cells form the background

Growth is slow and colonies may appear only in one to three weeks.

Epidemiology: Dermatophytosis occurs throughout the world, but certain types of disease and some species of fungi show geographically restricted distribution. Social and cultural patterns also influence dermatophytoses. *Tinea pedis*, so common in the temperate climates where all wear shoes, is rare in the tropics where most walk barefooted. Many factors, such as age, hormones and intercurrent diseases, affect the susceptibility to dermatophytosis.

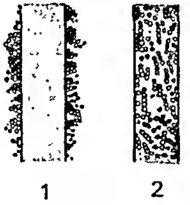

Fig. 65.6 Ectothrix and endothrix types of hair infection
1 ectothrix type with fungal arthrospores surrounding hair
2 endothrix type showing arthrospores inside hair shaft

Depending on their natural habitat, dermatophytes may be classfied as anthropophilic, zoophilic and geophilic species. Man is the main or only host for anthropophilic dermatophytes. *T. rubrum*, *E. floccosum* and *M. audouinii* are examples. They cause mild but chronic lesions. Zoophilic species are natural parasites of animals. Examples are *T. verrucosum* in cattle and *M. canis* in dogs and cats. Human infections with zoophilic dermatophytes cause severe inflammation, but are more readily curable. Geophilic species, which occur naturally in soil are relatively less pathogenic for man. Examples are *M. gypseum* and *T. ajelloi*.

Treatment: Topical antifungal agents are usually effective *T. rubrum* infections may be resistant to treatment. Oral griseofulvin is the drug of choice.

Candidosis

Candidosis (candidiasis, moniliasis) is an infection of the skin, mucosa, and rarely of the internal organs, caused by a yeast-like fungus *Candida albicans*, and occasionally, by other Candida species.

Candida albicans is an ovoid or spherical budding cell, which produces pseudomycelia both in culture and in tissues (Fig 65.7). Candida species are normal inhabitants of the skin and mucosa. Candidosis is an opportunist endogenous infection, the commonest predisposing factor being diabetes.

Cutaneous candidosis may be intertriginous or paronychial. The former is an erythematous, scaling or moist lesion with sharply demarcated borders, where papular lesions are most prominent. The sites affected are those where the skin is macerated by perspiration — groin, perineum, axillae and inframammary folds. Paronychia and onychia are seen in occupations that lead to frequent immersion of the hands in water.

Common mucosal lesions are vaginitis characterised by an acidic discharge and found frequently in pregnancy, and oral thrush found commonly in bottlefed infants and the aged and debili-

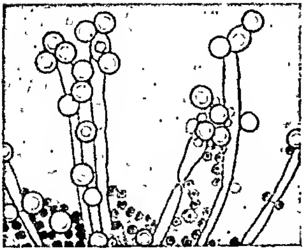

Fig 65 7a Yeasts and chlamydospores of *Candida albicans*

Fig 65 7b *Candida albicans* in a stained specimen of sputum The presence of hyphal elements in addition to yeast forms indicates colonisation of tissue by the organism.

tated. Creamy white patches appear on the tongue or buccal mucosa, that leave a red oozing surface on removal.

Intestinal candidosis is a frequent sequel to oral antibiotic therapy and may present as diarrhoea not responding to treatment. Bronchopulmonary candidosis is seen as a rare complication of preexisting pulmonary or systemic disease. Systemic infections, such as septicaemia, endocarditis and meningitis may occur as terminal complications in severe generalised diseases such as leukaemia and in persons on prolonged immunosuppression. Candida granuloma and chronic mucocutaneous candidiasis are serious manifestations seen in immunodeficiencies.

Diagnosis can be established by microscopy and culture. Wet films or Gram stained smears from lesions or exudates show budding Gram positive cells. As candida can be seen on normal skin or mucosa as well, only their abundant presence is of significance. Demonstration of mycelial forms indicates colonisation and tissue invasion and is, therefore, of greater significance. Cultures can be obtained readily on Sabouraud's and on ordinary bacteriological culture media Colonies are creamy white, smooth and with a yeasty odour. *Candida albicans* can be identified from other Candida species (*C. stellatoidae, C. tropicalis, C. pseudotropicalis, C. krusei, C. guilliermondii, C. parapsilosis, C. viswanathii*) by growth characteristics and sugar assimilation and ferment :ion tests. *C. albicans* alone forms chlamydospores on corn meal agar cultures at 20°C. A rapid method of identifying *C. albicans* is based on its ability to form germ tubes within two hours when incubated in human serum at 37°C (Reynolds-Braude phenomenon).

Agglutinins appear in the sera of patients, but as they are frequent in normal persons also, they are not helpful in diagnosis. Delayed hypersensitivity to candida is so universal that skin testing with Candida extracts is used as an indicator of the functional integrity of cell mediated immunity.

Management of candidosis is mainly by removing the predisposing causes. All Candida strains are sensitive to Nystatin, but as it is poorly absorbed from the gut, it is not useful in systemic diseases. Amphotericin B, 5-fluorocytosine and clotrimazole may be used for disseminated candidosis.

DEEP MYCOSES

Deep mycotic infections may be classified as

those that affect mainly or exclusively the subcutaneous tissues (subcutaneous or intermediate mycosis) and those that involve internal organs (deep seated or systemic mycoses).

Subcutaneous mycoses:

1. Mycotic mycetoma
2. Chromoblastomycosis
3. Sporotrichosis
4. Rhinosporidiosis
5. Subcutaneous phycomycosis

Systemic mycoses:

1. Cryptococcosis
2. Blastomycosis
3. Paracoccidioidomycosis
4. Coccidioidomycosis
5. Histoplasmosis

Mycetoma

Mycetomas are chronic, slowly progressive infections of the subcutaneous tissue, usually of the foot and rarely of the other parts of the body. The disease was originally reported by Gill (1842) from Madurai, South India, and Carter (1860) established its fungal aetiology. It is therefore commonly known as Maduramycosis or Madura foot. However, this conditon had been referred to in the Atharva Veda as Pādavālmīka (Foot anthill). It is seen mainly in the tropics, though occasional cases have been reported from temperate countries. Its incidence varies markedly from one place to another; for instance in India, it is quite common in Tamil Nadu, but rare in Kerala.

Mycetomas may be caused by a number of actinomycetes and filamentous fungi. A similar condition called 'botryomycosis' is caused by *Staphylococcus aureus* and some other bacteria. Aetiological diagnosis, therefore, is of importance in treatment.

The causative agent is believed to enter through minor trauma. The disease usually begins as a small subcutaneous swelling of the foot, which enlarges, burrowing into the deeper tissues and tracking to the surface as multiple sinuses discharging viscid, seropurulent fluid containing granules. These 'granules' or 'grains' are microcolonies of the aetiological agents and their demonstration is of diagnostic value. The colour and consistency of the grains vary with the different agents causing the disease (Table 65.3). In actinomycotic mycetoma, the grains will be composed of very thin (less than 1 μ in diameter) filaments, while in mycotic lesions, they will be broader and often show septae and chlamydospores. Actinomycotic lesions may respond to sulphonamides and antibiotics, but mycotic lesions are resistant and may require amputation

Chromomycosis

The term chromomycosis includes a group of clinical manifestations caused by various dematiaceous (pigmented) fungi.

1. *Chromoblastomycosis:* The most common

TABLE 65 3

Colour of grains in mycetomas of various aetiology

Colour of grain		
White to yellow	Brown to black	Red
Nocardia asteroides	*Madurella mycetomi*	*Actinomadura pelletieri*
Nocardia brasiliensis	*Madurella grisea*	
Actinomadura madurae	*Phialophora jeanselmei*	
Streptomyces somaliensis		
Allescheria boydii		

form of chromomycosis is known as chromoblastomycosis or verrucous dermatitis. The lesions consist of warty cutaneous nodules which resemble the florets of cauliflower. The disease is usually confined to the subcutaneous tissue of the feet and lower legs.

The aetiological agents are soil inhabiting fungi of the family Dematiaceac. They gain entrance through the skin by traumatic implanation. The lesion develops slowly around the site of implantation. The most common fungi responsible are *Fonsecaea (Hormodendrum)* species — *F. pedrosoi, F. compactum, F. dermatitidis; Phialophora* species *P. verrucosa* and *Cladosporium* species — *C. carrionii*. Infections caused by *F. pedrosoi* and *P. verrucosa* have been reported to disseminate to other areas, especially brain.

Histologically, the lesions show the presence of the fungus as round or irregular, dark brown, yeast-like bodies with septae, called *sclerotic cells* (Fig. 65.8). Diagnosis can be established by demonstration of these sclerotic bodies in KOH mounts or in sections, and by culture on Sabouraud's agar.

The disease is mainly tropical and is more common among barefooted agricultural workers and wood cutters.

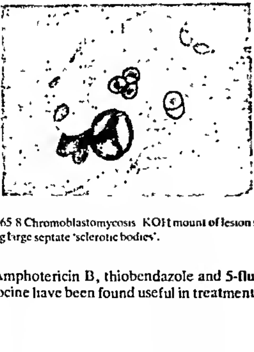

Fig 65 8 Chromoblastomycosis KOH mount of lesion showing large septate 'sclerotic bodies'.

Amphotericin B, thiobendazole and 5-fluorocytocine have been found useful in treatment.

2. *Other infections caused by dematiaceous fungi (Phaeohyphomycosis):* This group includes localised or systemic infections caused by certain species *Phialophora, Cladosporium* or other dematiaceous soil fungi, showing brown filaments in the affected tissues. The sites of lesions may be cutaneous, subcutaneous, deeper tissues, or organs like brain or lung. The tissue reactions and morphology of the fungus in lesions differ from those seen in chromoblastomycosis. Sclerotic cells or granules are not found. The fungi appear in lesions as distorted hyphal strands. Phaeohyphomycosis is generally seen in debilitated or immunodeficient hosts. Some of the clinical types are:

1. Brain abscess caused by *Cladosporium bantianum*, and
2. Subcutaneous or intramuscualr lesions with abscesses or cysts containing masses of brown hyphae (formerly known as *phaeosporotrichose)* caused by *Phialophora jeanselmei, P. spinifera, P. dermatitidis* or *P. richardsiae*

Sporotrichosis

Sporotrichosis is caused the fungus *Sporothrix (Sporotrichum) schenckii* and is characterised by the development on the skin, in subcutaneous tissues and in lymph nodes, of nodules which soften and break down to form indolent ulcers. The fungus is a saprophyte found widely, on plants, thorns and timber. Infection is acquired through thorn pricks or other minor injuries. Rare instances of transmission from patients and infected horses and rats, have been recorded. The disease is worldwide, though most cases occur in the USA.

The fungus spreads from the primary site through lymphatics, but seldom extends beyond the regional lymph nodes. Most cases occur in the upper limb. In infected tissues, the fungus is seen as cigar shaped yeast cells, without mycelia. Sometimes 'asteroid bodies' are seen in the lesion, composed of a central fungus cell with eosinophilic material radiating from it.

Diagnosis is made by culture as frequently the

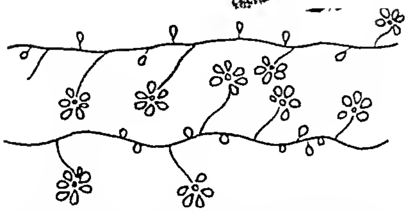

Fig 65.9 *Sporothrix (Sporotrichum) schenckii* culture mount showing fine branching hyphae and pear shaped conidia borne in rosette like clusters at tips of lateral branches and singly along sides of hyphae

fungus may not be demonstrable in pus or tissues. *S. schenckii* is a dimorphic fungus occurring in the yeast phase in tissues and in cultures at 37°C, and in the mycelial phase in nature and in cultures at room temperature. The septate hyphae are very thin (1–2 μ diameter) and carry flower-like clusters of small conidia borne on delicate sterigmata (Fig. 65.9). Rats are highly susceptible and can be infected by intraperitoneal or intratesticular inoculation

Rhinosporidiosis

Rhinosporidiosis is a chronic granulomatous disease characterised by the development of friable polyps, usually confined to the nose, mouth or eye, but rarely seen on the genitalia or other mucous membranes. Though the disease was first identified in Argentina, the large majority of cases come from India and Sri Lanka. While the disease is generally confined to mucous membranes, haematogenous dissemination has been recorded very rarely.

Histologically the lesion is composed of large numbers of fungal spherules embedded in a stroma of connective tissue and capillaries. The spherules are 10–200 μ in diameter and contain thousands of endospores (Fig. 65.10).

The causative fungus *Rhinosporidium seeberi* has not been cultivated. The mode of infection is not known though infection is believed to originate from stagnant water or aquatic life.

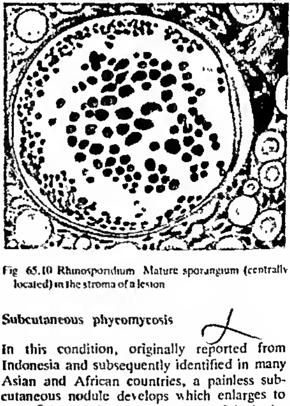

Fig 65.10 Rhinosporidium Mature sporangium (centrally located) in the stroma of a lesion

Subcutaneous phycomycosis

In this condition, originally reported from Indonesia and subsequently identified in many Asian and African countries, a painless subcutaneous nodule develops which enlarges to involve a whole limb or large areas of the body. The causative agent is *Basidiobolus haptosporus*,

a saprophytic phycomycete found in decaying vegetation and in the intestines of many reptiles and amphibians. It has been suggested that the infection may be acquired by insect bites.

Cryptococcosis

Cryptococcosis is a subacute or chronic infection caused by the yeast *Cryptococcus neoformans*. It is a round or ovoid budding cell, 4–20μ in diameter, with a prominent polysaccharide capsule (Fig. 65.11). It is a soil saprophyte and is particularly abundant in the faeces of pigeons and other birds.

Infection is usually acquired by inhalation, but may sometimes be through skin or mucosa. Most infections are asymptomatic. Pulmonary cryptococcosis may lead to a mild pneumonitis. As no calcification occurs, healed pulmonary lesions are not evident radiologically. Dissemination of infection leads to visceral, cutaneous and meningeal disease. Visceral forms simulate tuberculosis and cancer clinically. Bones and joints may be involved. Cutaneous cryptococcosis varies from small ulcers to large granulomas. Cryptococcal meningitis is the most serious type of infection and can resemble tuberculous or other chronic types of meningitis. Its onset is insidious and the course slow and progressive.

Diagnosis is established by demonstration of capsulated, budding yeast cells in the lesions and by culture. The capsules stand out in Indian ink preparations. The fungus grows readily on Sabouraud's agar forming smooth, mucoid, cream coloured colonies. The ability to grow at 37°C and hydrolyse urea differentiates *C. neoformans* from nonpathogenic cryptococci. Pathogenicity can be demonstrated by intracerebral or intraperitoneal inoculation into mice, which develop a fatal infection. Capsulated budding yeast cells can be demonstrated in the brain of infected mice.

Two perfect stages of the fungus have been discovered. They belong to the class Basidiomycetes and have been termed *Filobasidiella neoformans* and *F. basilispora*.

Four serological types of cryptococcal capsular polysaccharide — A, B, C and D — have been identified. Demonstration of the capsular antigen by precipitation can sometimes be valuable in diagnosing some cases of cryptococcal meningitis, when the CSF is negative by smear and culture. Amphotericin B, 5-fluorocytosine, clotrimazole and miconazole are useful in the treatment of the disease.

Cryptococcosis is worldwide in distribution. As it was originally reported from Europe, it used to be known as 'European blastomycosis'. Sev-

Fig. 65.11 *Cryptococcus neoformans*. Indian ink preparation of spinal fluid showing yeast cells surrounded by a large capsule.

eral cases of cryptococcosis have been identified in India, this being the only deep mycosis common in this country. *C. neoformans* can produce disease in animals, particularly mastitis in cattle. The role of animal cryptococcosis in causing human disease is not known

Blastomycosis

This is a chronic infection caused by the dimorphic fungus *Blastomyces dermatitidis*, characterised by the formation of suppurative and granulomatous lesions in any part of the body, but with a marked predilection for the lungs and skin. As the infection is largely confined to the North American continent, it is known as the 'North American blastomycosis'. A number of cases have however been reported from Africa lately. The fungus has also been isolated in Delhi from bronchial aspirates of a patient and from the lungs of insectivorous bats.

Soil is considered to be the source of infection, which is acquired by inhalation. Primary infection of the lung may resemble tuberculosis or histoplasmosis. It may be asymptomatic or may lead to focal or diffuse consolidation, miliary lesions or abscess formation. The fungus may spread from the lungs through the bloodstream and form multiple abscesses in various parts of the body. Case fatality is high in the generalised disease. The cutaneous disease is usually on the skin of the face or other exposed parts of the body. The initial lesion is a papule, around which secondary nodules develop and coalesce, leading to large, elevated ulcerative lesions.

In tissue and in cultures at 37°C, the fungus appears as budding yeast cells, which are large (7–20 μ) and spherical, with thick, double contoured walls. Each cell carries only a single broadbased bud (Fig. 65.12). At room temperature, the culture is filamentous with septate hyphae and many round or oval conidia, and in older cultures chlamydospores also.

Paracoccidioidomycosis

This is a chronic granulomatous disease of the skin, mucosa, lymph nodes and internal organs caused by *Paracoccidioides brasiliensis*. As the disease is confined to South America, it is called 'South American blastomycosis'. Ulcerative granulomas of the buccal and nasal mucosa are a prominent feature of the disease.

The yeast phase is found in tissues and in cul-

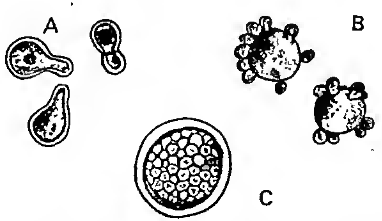

Fig. 65.12 A *Blastomyces dermatitidis* yeast phase showing spherical or oval double walled cells with single broad based buds. B *Paracoccidioides brasiliensis* yeast phase showing spherical thick walled cells with multiple buds, C *Coccidioides immitis* tissue phase showing spherule with numerous endospores

tures at 37°C. It consists of large, round or oval cells with multiple budding (Fig.65.12). The mycelial phase develops at room temperature.

Coccidioidomycosis

Coccidioidomycosis is an infection caused by the dimorphic fungus *Coccidioides immitis*. The infection may be inapparent, benign, severe or even fatal. The disease is endemic in the dry, arid regions of Southwestern USA, where the fungus is present in the soil and in rodents.

Infection is acquired by inhalation of dust containing arthrospores of the fungus. In most cases, the respiratory infection is asymptomatic and leads only to immunisation, which can be demonstrated by a positive skin test with 'coccidioidin' (analogous to the tuberculin test). Many persons develop a self-limited influenza-like fever (known as 'valley fever' or 'desert rheumatism'). Less than one per cent of infected persons develop chronic progressive disseminated disease (coccidioidal granuloma) which is highly fatal. It resembles clinically and histologically disseminated tuberculosis.

The fungus is dimorphic, occurring in the tissue as a yeast and in culture (both at 37°C and at room temperature) as the mycelial form. The tissue form is a spherule, 15–75μ in diameter, with a thick doubly refractile wall and filled with endospores (Fig. 65.12). The mycelial phase consists of hyphae which fragment into arthrospores which are highly infectious. Cultures should be handled with particular care as they may cause laboratory infection.

Histoplasmosis

Histoplasmosis is an intracellular infection of the reticuloendothelial system caused by the dimorphic fungus *Histoplasma capsulatum*. The disease was originally described by Darling (1905) who believed the causative agent to be a protozoon related to *Leishmania donovani*.

The disease is worldwide in distribution but is most common in the USA where it is endemic in many central and eastern states. In endemic areas the fungus is present in the soil, rotting trees and is particularly abundant in bird faeces. Infection is acquired by inhalation. The large majority of infections are asymptomatic and, as in tuberculosis, heal, leaving behind an area of miliary calcification. It was the investigation of tuberculin negative individuals with pulmonary calcification that made evident the frequency of asymptomatic infection with the fungus. Some infected persons develop pulmonary disease which resembles tuberculosis. Disseminated histoplasmosis develops only in a small minority of infected individuals. The reticuloendothelial system is involved with resultant lymphadenopathy, hepatosplenomegaly, fever, anaemia and a high rate of fatality. Granulomatous and ulcerative lesions may develop on the skin and mucosa.

In tissues, the fungus is present inside phagocytic cells in the yeast phase — oval, budding cells measuring 2–4μ. The yeast phase is also formed in blood agar cultures at 37°C On Sabouraud's agar at room temperature, white cottony mycelial growth appears, with large (8–20μ) thick walled, spherical spores with tubercles or finger-like projections (Fig. 65.13). The appearance of the tuberculate spores is diagnostic.

Diagnosis may be made by microscopical examination of stained smears of blood, bone marrow, scrapings from lesions or biopsies of lymph nodes, and by the culture of the fungus from these materials. Antibodies are formed during the infection. They decline if the infection is inactive, but increase in titre in progressive disease. Latex agglutination, complement fixation and precipitation tests are useful in diagnosis. Delayed hypersensitivity is developed following infection. It can be demonstrated by skin testing with 'histoplasmin', which is analogous to the tuberculin test for tuberculosis.

'African histoplasmosis' involves mainly the skin, subcutaneous tissues and bones. The lungs are not commonly affected and disseminated disease is infrequent. The causative agent has been named *Histoplasma duboisii* and it differs from *H. capsulatum* in forming much larger yeast-like

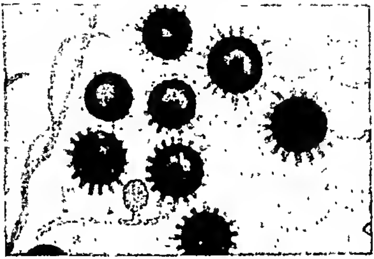

Fig. 65.13 *Histoplasma capsulatum*

cells (7–15μ). In the mycelial form, the two species are indistinguishable.

Amphotericin B has been found useful in therapy.

OPPORTUNISTIC SYSTEMIC MYCOSES

Some saprophytic fungi which are ubiquitous in the environment are important in medical mycology for two reasons. Firstly, they are common laboratory contaminants on culture media — *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* species grow on virtually anything. Secondly, they can produce serious and even fatal infection in persons who are otherwise debilitated. Aspergillosis and mucormycosis are important opportunistic systemic mycoses.

Aspergillosis

Aspergilli and Penicillium constitute the commonest moulds seen on damp bread or almost any other organic matter. Of the 300 odd species of aspergilli, *A. fumigatus* is highly pathogenic for birds, and occasionally causes invasive disease in man. A few other species may also cause opportunistic human disease. The commonest human disease caused by aspergilli is otomycosis.

Systemic aspergillosis occurs as the following clinical types:

1. Pulmonary aspergillosis
   a. Aspergillus asthma
   b. Bronchopulmonary aspergillosis
   c. Colonising aspergillosis (Aspergilloma)
2. Disseminated aspergillosis

Aspergillus asthma occurs in atopic individuals following sensitisation to inhaled aspergillus spores. In bronchopulmonary aspergillosis, the fungus grows within the lumen of the bronchioles, which may be occluded by fungus plugs. The fungus can be demonstrated in sputum. The condition is made worse by the development of hypersensitivity to the fungus. Colonising aspergillosis usually develops in preexisting pulmonary cavities, such as in tuberculosis or cystic disease. The fungus grows into large 'balls' (Aspergilloma). Surgical removal becomes necessary as the disease commonly causes massive haemoptysis. In invasive aspergillosis, the fungus actively invades the lung tissue. Disseminated aspergillosis involving the brain, kidney and other organs is a fatal complication sometimes seen in debilitated patients on prolonged treatment with antibiotics, steriods and cytotoxic drugs.

Diagnosis may be made by microscopical examination and by culture. The fungus grows

rapidly on culture media. Identification of Aspergillus is easy, based on growth characteristics and morphology. *Aspergilli* have septate byphae. Asexual conidia are arranged in chains, carried on elongated cells called 'sterigmata', borne on the expanded ends (vesicles) of conidiophores (Fig. 65.14).

As aspergilli are such common contaminants, their demonstration in exudates and isolation in cultures have to be interpreted with care.

Penicilliosis

Penicillium species have been very rarely incriminated in opportunistic human infections. Members of this genus can be identified by the brush-like arrangement of conidia (Fig.65.14).

Mucormycosis

Mucormycosis is an invasive disease caused by phycomycetes, mainly by species of Rhizopus, Mucor and Absidia. It used to be a rare terminal complication of uncontrolled diabetes and other chronic debilitating diseases. The incidence of the disease has increased considerably as a result of the widespread use of antibiotics, steroids and antimetabolites. The fungi are normally avirulent and are able to invade tissues only when general resistance is extremely low.

The primary infection is usually in the upper respiratory tract or nose, where the spores germinate and the mycelia invade the adjacent tissues — the orbit, sinuses and the brain. Primary infection may also occur in the lung, the fungi invading the arteries to cause thrombosis and infarction. The disease is fatal.

Diagnosis is usually made during histological examination of autopsy material, by the presence of broad, nonseptate mycelia in tissues. The fungi can be grown easily on Sabouraud's medium without cycloheximide. Mucor shows branched sporangiophores arising randomly along aerial mycelium. Rhizoids are absent. Rhizopus has rhizoids, and sporangiophores arise in groups directly above the rhizoids (Rig. 65.14).

Otomycosis

Otomycosis is fungal infection of the external ear. It is a very common disease and is usually caused by species of aspergilli (*A.niger, A. fumigatus*) and penicillia. The symptoms are itching, pain and deafness. Secondary bacterial infection, commonly due to Pseudomonas and Proteus causes suppuration. Diagnosis can be made by demonstration of the fungi in scrapings and by culture.

Oculomycosis

Mycotic keratitis usually follows corneal trauma. Fungal spores colonise the injured tissue and initiate an inflammatory reaction, leading to hypopyon ulcer and endophthalmitis. If not recognised and treated early, enucleation may become necessary. The widespread use of corticosteriods in ophthalmology has resulted in an increased incidence of keratomycosis.

Many saprophytic fungi can cause ocular infection, Aspergillus species, Fusarium and *Candida albicans* being most often responsible. Diagnosis may be made by examination of deep scrapings. Superficial swabs may not show the fungus. Local application of amphotericin B, Nystatin and Pimaricin (Natamycin) may be useful.

Mycotic poisoning

Many fungi form poisonous substances. Mycotic poisoning is of two types — *mycetism* in which a fungus which is eaten for itself causes toxic effects and *mycotoxicosis* in which fungal toxins contaminate some article of food.

Mycetism has been known from ancient times, several varieties of poisonous mushrooms having been identified as inedible. Mycetism may cause gastrointestinal disease, dermatitis or death. The hallucinogenic agents (d-lysergic acid, psilocybin) produced by *Psilocybe* species and other fungi have attracted much attention in recent years.

The best known mycotoxin is 'Aflatoxin' pro-

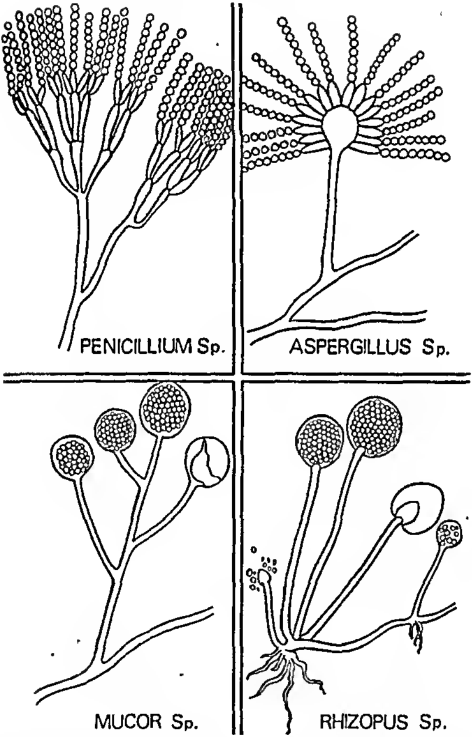

Fig. 65.14 Common contaminant fungi, culture mounts. *Penicillium* showing the 'penicillus' or brush, consisting of chains of spores extending from the ends of short branches of conidiophores. *Aspergillus* showing unbranched nonseptate con-[illegible]ores terminating in a globose vesicle bearing phialids from which arise chains of conidia. *Mucor* showing nonseptate [illegible]um without rhizoids (root-like-structures). Sporangiophores, which may be branched, terminate in large globose [illegible]ngia containing numerous spores. *Rhizopus* showing nonseptate mycelium with rhizoids. Unbranched sporangiophores [illegible]e opposite rhizoids.

duced by *Aspergillus flavus.* It is frequently present in mouldy foods, particularly in groundnuts, corn and peas. It is highly toxic to animals and birds, and probably to man as well. It can cause hepatomas in ducklings and rats, and its possible carcinogenic effect in man is of great concern. There have been several reports of aflatoxicosis from India, involving man and animals.

Ergotoxicosis (ergotism) is due to the toxic alkaloids produced by the fungus *Claviceps purpurea,* while growing on the fruiting heads of rye.

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66 Acute Diarrhoeal Diseases

Acute diarrhoeal diseases constitute an important cause of morbidity and mortality throughout the world, particularly in infants and children in the developing countries. It has been estimated that some 500 million episodes of diarrhoea occur annually among children under five years of age in the developing countries of Asia, Africa and Latin America, causing 5–18 million deaths. In India, 1.4 million children die annually from diarrhoeal diseases other than cholera. Apart from causing death due to dehydration, diarrhoeal diseases often initiate malnutrition, which is aggravated by each successive episode. Diarrhoeal diseases are important even in the affluent countries. In the USA, for instance, diarrhoea is second only to acute respiratory disease as a cause of absenteeism from work, and ranks among the five leading causes of death in young children.

Acute diarrhoeal diseases vary in severity from a passing inconvenience to a rapidly fatal flux. While frank diarrhoea is self-evident, mild diarrhoea is difficult to define as it is dependent on individual bowel habits, which are very variable. Diarrhoea may be defined as an increase in the frequency, fluidity or volume of bowel movements relative to the usual habits of each individual. As a rough guide, passage of three or more motions a day can be taken as diarrhoea. The causes of diarrhoea are legion, ranging from infections to emotional factors. However, the large majority are due to the action of microorganisms or their products in the intestines and only these are considered here.

Diarrhoeal diseases present several epidemiological patterns. Based on the age groups involved, they may be classified as infantile, weanling, childhood or adult diarrhoeas. Some types present seasonal patterns, occurring during summer, winter or monsoon. The disease may be sporadic, endemic or pandemic. Certain types may be place associated, as for example the so called asylum diarrhoea. The term gastroenteritis is often used as a synonym for acute diarrhoea, especially when associated with vomiting. Though the term gastroenteritis may not be semantically correct where inflammation is absent, it has the sanction of long usage. The term dysentery denotes passage of blood and mucus with motion, often with tenesmus. Travellers' diarrhoea is an acute diarrhoeal illness that sometimes occurs in visitors from foreign countries, within a week or two of arrival in a developing country such as India, Bangladesh or Mexico. This condition used to be referred to by such exotic names as Delhi belly and Montezuma's revenge. The term 'food poisoning' may mean any type of illness acquired through consumption of food or drink contaminated with microorganisms, their toxins or chemical poisons. But traditionally, it is restricted to acute diarrhoea, with or without vomiting caused by microbial contamination of food.

Diarrhoea involves increased loss of fluid and electrolytes from the intestine. About eight litres of water enter the duodenum every day, consisting of one litre of saliva, two of gastric juice, two of pancreatic juice and one of bile, together with an average of two litres in food and drink. In the small intestine further fluid is added from the plasma, but absorption is so efficient that only 500–1000 ml of water enter the caecum from the terminal ileum. In the large bowel also, some

The Vats

water is secreted into the lumeo, but net absorption results in only some 100 ml appearing in stools as faecal water normally. Several mechanisms control the secretion and absorption of water and electrolytes io tbe gut. Examples of mechanisms that promote absorption are the sodium pump, luminal glucose, amino acids or electrolytes, catecbolamine action mediated by the cyclic GMP, and steroids. Examples of mechanisms that promote secretion are cyclic AMP, pitressin, secretin, glucagon, bacterial enterotoxins, fatty acids, bile acids, undigested disaccharides, gastric acid and prostaglandin. Alteration of the complex balance between these factors results in diarrhoea. Diarrhoeal stool is usually isotonic with plasma, but may differ greatly in electrolyte content. The amount of electrolytes lost depends on various factors such as the aetiology of diarrhoea and the age of the patient.

Acute diarrhoea due to bacterial infection can be classified into three groups based on pathogenic mechanisms. The first group, exemplified by cholera vibrio and enterotoxigenic *E.coli*, produces diarrhoea by multiplying in the small intestine and secreting eoterotoxins which cause profuse secretion of fluid and electrolytes. The bacteria do not invade the bowel mucosa, which appears histologically normal. Systemic symptoms such as fever are minimal or absent. The second type is represented by shigellae and enteroinvasive *E.coli*. They invade the bowel mucosa. The pathology is initially in the small intestine, when the patient develops abdominal cramps, mild fever and watery diarrhoea. In a day or two, the large bowel is invaded, with colitis leading to urgency, tenesmus and bloody mucoid stools typical of dysentery. The third group is typified by salmonellae. The bacteria penetrate the gut mucosa and reach the lamina propria where they set up inflammation. Bacteraemia and systemic symptoms such as fever are very common. In general, profuse watery diarrhoea is seen in enterotoxic infections such as cholera, as well as in rotaviral diarrhoea. Invasive diarrhoea leads to liquid stools rich in cellular exudate.

In addition to the above mechanisms of infection, diarrhoea can also occur due to the action of preformed bacterial toxins, as in the case of staphylococcal food poisoning. Here bacterial multiplication in the gut is not necessary.

AETIOLOGY OF DIARRHOEAL DISEASES

A large number of microorganisms are now known to cause diarrhoea. Until recently, the causative agents could be identified in only 10–20 per cent of diarrhoeal illnesses. In the last decade, additional pathogens have been recognised so that now, under optimal laboratory conditions, aetiological diagnosis can be established in some 80 per cent of cases. The bacterial, viral, protozoal and fungal agents responsible for acute diarrhoeal illness are listed below (Table 66.1).

A. Bacteria

1. Vibrios: a) *V. cholerae: V. cholerae*, classical as well as el Tor biotypes, cause cholera, the most spectacular of diarrhoeal diseases. The vibrio enters the intestines through food or drink. The infective dose is large, about 10^8 cells. The site of infection is the small intestine, primarily the jejunum, where it multiplies and elaborates the enterotoxin. Pathogenicity depends on the ability of the vibrio to adhere to intestinal epithelium and colonise in the gut, as well as on the production of the toxin.

Cholera is endemic in Bangladesh, West Bengal and many other parts of India. In endemic areas it is predominantly a disease of children. When it appears in nonendemic areas, it affects all ages, most commonly adult males. In both endemic and epidemic situations, cholera exhibits seasonal patterns, which vary in different areas.

Cholera can occur in varying grades of severity, from a rapidly fatal disease to a mild diarrhoea. Asymptomatic infection is common with el Tor vibrios. Prolonged gall bladder carriage may occur very rarely. There is increasing evi-

TABLE 66 1

A summary of important characteristics of infective diarrhoeal diseases

Causative agent	Reservoir	Mode of transmission	Pathogenesis	Main features
		BACTERIAL		
Vibrio cholerae	Man ? Marine life	Water, food. Rarely Person to person	Enterotoxin	Severity ranging from acutely fatal to mild watery diarrhoea; seasonal pattern: mainly affects children in endemic areas and adults in nonendemic areas; causes epidemics and pandemics.
Noncholera vibrios (NAG)	Water, marine life, man.	Water, food	?Enterotoxin	Mild diarrhoea to cholera-like disease.
Vibrio parahaemolyticus	Sea water, marine life.	Sea fish, prawns, crabs	? Enterotoxin ? Invasive	Mild watery diarrhoea or dysentery-like illness; sporadic diarrhoea or food poisoning.
Enterotoxigenic *E. coli*	Man	Food, water.	Enterotoxins, LT and ST	Watery diarrhoea; common in young children in endemic areas; Important cause of 'Travellers' diarrhoea'
Enteropathogenic *E. coli*	Man	Person to person	? Enterotoxin.	Associated with institutional outbreaks in infants; significance in sporadic diarrhoea uncertain, infrequent in developing countries.
Enteroinvasive *E. coli*	Man	Food	Invasive	Dysentery-like illness; strains identified by Sereney test.
Enterohaemorrhagic *E. coli*	Man	Food	Invasive	Haemorrhagic colitis
Salmonellae (about 2000 serotypes)	Animals and birds (*S. typhi* in man.)	Food; Institutional outbreaks	Invasive (? Enterotoxin)	Food poisoning from animal foods or food products; febrile gastroenteritis.
Shigellae (4 serogroups)	Man	Person to person; water, food.	Invasive (? Enterotoxin)	Dysentery; watery diarrhoea can also occur; *Sh. flexneri* commonest in developing countries, large epidemics by multiresistant *Sh. dysenteriae I*
Cl perfringens	Human and animal intestine	Food	Enterotoxin	Food poisoning, Type A strains commonly responsible; Type C strains cause necrotising enteritis.

Bacillus cereus	Soil	Food. Especially rice and grains	Enterotoxins	Two types of food poisoning, — one primarily vomiting, the other primarily diarrhoea.
Staph. aureus	Man	Food, especially meat and milk products.	Enterotoxins	Toxic type of food poisoning; primarily *vomiting*, and *diarrhoea*.
Campylobacter jejuni	Domestic animals, birds.	Food, water; from animals and birds, ? man to man	? Invasive	Febrile gastroenteritis with colicky abdominal pain.
Yersinia enterocolitica	Man, animals.	Food, water, ? man to man ? animal to man	? Invasive ? Toxin	Clinical syndrome varies with age; febrile diarrhoea; mesenteric adenitis; common in cold climates.
		VIRAL		
Rotavirus	Man	Person to person	Invasive	Worldwide, common in winter; Infection asymptomatic in *infants and adults*; Peak incidence 6–24 months of age. Vomiting common.
Norwalk virus	Man (? shell fish)	Person to person; ? from oysters and cockles	Invasive	More common in older children and adults
		PARASITIC		
Entamoeba histolytica	Man	Food, water; person to person	Invasive	Dysentery; leads to chronic amoebiasis and extra intestinal manifestations.
Giardia lamblia	Man, animals	Food, water; person to person.	? Invasive	Watery, loose or fatty stools; May lead to persistent diarrhoea and malabsorption; causes one type of travellers' diarrhoea.
Cryptosporidium	Man, Animals, Birds	Food	Invasive	Diarrhoea in immunodeficient
Balantidium coli	Man, Pigs	Food	Invasive	Subacute to chronic diarrhoea
		FUNGAL		
Candida albicans	Man	endogenous infection.	?	May be primary or iatrogenic following *oral antibiotics*.

dence that el Tor vibrios can survive for long periods in aquatic environments.

b) *Noncholera (NAG) vibrios:* These vibrios resemble cholera vibrios except for being inagglutinable with O group antiserum. The pathogenic status of NAG vibrios is not clear. Some of them have caused outbreaks of cholera-like disease, while most of them appear to be nonpathogenic, having been frequently observed in natural water sources and in normal stools. A few strains have been shown to produce a heat labile enterotoxin resembling the cholera toxin.

c) *V. parahaemolyticus:* First isolated in Japan in the 1950's from outbreaks of food poisoning, this halophilic vibrio has subsequently been demonstrated in the coastal waters of several countries. Several O and K antigenic types of the vibrio exist, but there is no association with pathogenicity and any particular serotype. Haemolysis on a special high salt agar (Kanagawa phenomenon) has been claimed to be useful in differentiating between pathogenic and non-pathogenic strains.

This vibrio can cause two types of clinical syndromes, the more common watery diarrhoea and the less common syndrome of bloody diarrhoea with abdominal cramps and fever. *V parahaemolyticus* has been responsible for the majority of food poisoning cases in Japan. This vibrio has been reported to be the causative agent in about 10 per cent of hospitalised diarrhoea patients in Calcutta, but elsewhere in India it does not appear to be important in diarrhoeal illness. Kanagawa negative vibrios are frequently found in fishes and crustaceans from the coastal waters, but human infection has been rare.

The mechanism of *V. parahaemolyticus* diarrhoea is not well understood. The vibrio has been reported to produce ballooning of the rabbit ileal loop and an enterotoxin has been postulated. The vibrio also causes local invasion of the bowel mucosa.

2. *Escherichia coli*: *E. coli* appears to be the most versatile of diarrhoeagenic agents. At least four mechanisms of pathogenesis have been identified in *E. coli* diarrhoea, different sets of strains being responsible for the different types of pathogenesis.

a) Enterotoxigenic *E. coli*. (ETEC): This is the type of *E. coli* believed to be responsible most often for diarrhoeal illness in the developing countries. ETEC produce a heat labile (LT), heat stable (ST) or both types (LT-ST) of enterotoxins. The LT activity is similar to that of cholera toxin, to which it is antigenically related. ST activity is believed to be through activation of guanyl cyclase in the small intestine. It is antigenically unrelated to cholera toxin. LT production can be demonstrated by ileal loop tests, tissue culture techniques or by ELISA, while ST production can be detected only by the cumbersome infant mouse intragastric assay. Toxin production is plasmid mediated. Pathogenicity also depends on the plasmid mediated colonisation factors, which are essential for attachment of the strains to intestinal mucosa.

ETEC cause mild diarrhoea to severe choleraic disease in the developing countries. They form the most important cause of travellers' diarrhoea. The relative frequency of LT, ST and LT-ST strains varies in different areas. ETEC are frequently responsible for diarrhoea in calves, but there is no evidence that human and animal strains are related.

b) Enteropathogenic *E. coli* (EPEC): From 1945, certain OB serotypes of *E. coli* have been recognised as responsible for diarrhoea in infants, particularly in nurseries. The pathogenesis of EPEC diarrhoea is not fully understood. It seems to depend on the ability of the strains to adhere to the gut mucosa and multiply. Some strains have been found to produce an enterotoxin.

EPEC diarrhoea has been declining in incidence during the last decade or so. As some of these serotypes are frequently found in normal stools, the significance of recovery of EPEC from sporadic diarrhoea is uncertain. EPEC are not considered to be an important cause of acute diarrhoeal disease in the developing countries.

c) Enteroinvasive *E. coli* (EIEC): These strains resemble shigellae in their properties and

pathogenicity. They are confined to a few O serogroups (Groups 28 ac, 112 ac, 124, 136, 143, 144, 152, 164). They may cause watery diarrhoea or typical dysentery.

Identification of EIEC depends on the Sereney test or on the invasion of cultured HeLa or Vero cells. Because of the difficulty in identifying them, the true prevalence of EIEC diarrhoea is not known. Cases of food poisoning due to EIEC have been reported.

d) Enterohaemorrhagic *E. coli* (EHEC): *E. coli* 0157:H7 has been found to cause haemorrhagic colitis. It forms a cytotoxin resembling the toxin of *Sh. shiga*.

3. Salmonellae: Some 2000 serotypes of salmonellae are known, any of which, with the possible exception of *S. typhi*, can cause acute diarrhoeal disease. But the large majority of outbreaks are caused by *S. typhimurium*, *S. enteritidis* and about a dozen other serotypes.

Salmonellae invade the ileal epithelial cells, penetrate to the lamina propria and usually initiate bacteraemia, with fever and other systemic manifestations. An enterotoxin has also been demonstrated in some serotypes, such as *S. typhimurium*. The pathogenic significance of the enterotoxin is not known.

Animals and birds constitute the natural reservoir of salmonellae. Human infection is usually zoonotic, caused by consumption of animal foods or food products. Salmonellosis is the commonest type of food poisoning in Britain and many other countries. Convalescent and asymptomatic human carriers are common, but chronic carriage is rare, being seen mainly in patients with chronic gall bladder disease.

Salmonellae frequently harbour plasmids, which confer resistance to multiple antibiotics. Plasmid bearing salmonellae often show enhanced virulence and communicability. Some of them exhibit altered ecological and epidemiological features, spreading directly from man to man, or fomites to man. *S.typhimurium* strains carrying R plasmids have become important hospital pathogens in many countries, causing serious systemic infections, particularly in the newborn.

4. Shigellae: All four serogroups of shigellae produce dysentery. The pathogenesis of dysentery depends on the penetration of epithelial cells of the large bowel and subsequent local multiplication, producing colitis. In addition, shigellae may also produce a mild watery diarrhoea. This is believed to be due to the action of an enterotoxin on the small bowel. All serogroups have been found to produce enterotoxin.

Shigellosis is worldwide, being more common in poor, overcrowded and unhygienic communities. It has been described as a 'water-washed disease', its incidence decreasing as the amount of water used for sanitation increases. *Sh. flexneri* is the commonest type in the developing countries and *Sh. sonnei* in the developed countries. *Sh. dysenteriae type I* causes the most serious types of dysentery. R factors are common in shigellae. A strain of *Sh. dysenteriae type I* carrying R plasmids has caused very extensive epidemics in recent years in many developing countries of South America, Africa and Asia, including India.

Shigellae are host specific for man. Caged monkeys frequently suffer from shigellosis, the infection being transmitted from their keepers. No extrahuman reservoir is known. Human carriers are common. Infection spreads by person to person contact, though water and food borne outbreaks also occur. The infective dose is small, as few as 10 bacilli being capable of causing the disease.

5. Clostridium perfringens: *Cl. perfringens (Cl. welchii)* is a spore forming bacillus widely distributed in soil and frequently found in the intestinal tract of man and animals. Five types are recognised, A to E. Most cases of human clostridial gastroenteritis are caused by type A. Certain heat resistant nonhaemolytic strains of type A have been recognised as an important cause of food poisoning. Various heat sensitive haemolytic strains have also been reported to cause diarrhoea.

In contrast to the nonfatal watery diarrhoea caused by type A strains, some type C strains can cause a severe haemorrhagic necrotising jejunitis, leading to bloody diarrhoea, severe abdominal pain and shock. Originally described in Germany under the name Darmbrand ('fire in the belly') this has subsequently been reported from New Guinea as 'Pigbel' and as necrotising enteritis from many other parts of the world.

Type A strains produce an enterotoxin, which appears to be a structural component of the spore coat. Type C strains also produce a related enterotoxin.

6. Bacillus cereus: This is a ubiquitous aerobic sporebearing bacillus. Ingestion of food heavily contaminated with *B. cereus* leads to diarrhoea. Two clinical syndromes are caused by this bacillus, one characterised by diarrhoea and abdominal cramps, and the other by nausea and vomiting, resembling staphylococcal food poisoning. Both types appear to be caused by enterotoxins.

7. Staphylococcus aureus: Some strains of *Staph. aureus* produce enterotoxin. When food gets contaminated with such strains, they multiply and elaborate the toxin, which is heat stable. Ingestion of such food results in vomiting and diarrhoea.

Six types of staphylococcal enterotoxins have been identified, types A to F. The majority of reported outbreaks have been caused by strains producing enterotoxin type A, or type A and D together.

8. Campylobacter jejuni: This microaerophilic bacterium, has been reported as responsible for some 5–15 per cent of diarrhoeal illness in England, Belgium and some other countries. The illness typically has a febrile prodrome for 12–24 hours, followed by watery diarrhoea and colicky abdominal pain. The disease may last for two weeks or more and is sometimes associated with vomiting and bloody mucoid stools.

Domestic animals and birds are considered to be the reservoirs Milk and waterborne outbreaks have been reported. Isolation is difficult, requiring special media with antibiotic supplements and incubation at 43°C under microaerophilic conditions. While campylobacter is a major cause of infective diarrhoea in many affluent countries, it appears to be much less important in India and other developing countries, where asymptomatic infection is common.

9. Yersinia enterocolitica: Formerly known as *Pasteurella pseudotuberculosis* this bacterium is now considered a member of the family Enterobacteriaceae. It has been identified as an important cause of diarrhoea in Europe, Japan and North America, especially in the cold season. The symptoms appear to be related to the age of the patients. Infants typically develop febrile diarrhoea, older children have acute mesenteric lymphadenitis, and adults tend to develop enteritis with fever, reactive arthritis and erythema nodosum. Some cases simulate acute appendicitis.

The bacillus has been isolated from swine and dogs, from foods and from natural water sources. Foodborne outbreaks have been recorded, but sporadic cases also occur. The bacillus grows optimally at 22°–29°C. It can survive for long periods and even grow at 4°C, a property that enables it to cause outbreaks of enteritis through contaminated food, particularly milk. Serotyping, biotyping and phage typing methods have been developed. Serotypes 03, 08 and 09 and biotypes 2, 3 and 4 are usually isolated from human cases. Some strains produce an enterotoxin resembling the ST toxin of *E.coli*. Some other strains have been found to be invasive by the Sereney test.

10. Other bacteria: Many other bacteria have been reported to cause diarrhoea. *Pseudomonas aeruginosa* was first implicated in diarrhoea as early as 1894. A number of cases have been recorded recently. Diarrhoeagenic strains have been shown to form an enterotoxin.

Aeromonas hydrophila and *Plesiomonas shigelloides* have been reported to cause diarrhoeal illness.

B. Viruses

Though viruses had for long been suspected to cause diarrhoea, it had not been possible to relate conventional viruses, which could be grown in tissue culture, with the illness. The importance of viruses in diarrhoea came to be recognised only from the discovery of rotavirus in 1973.

1. Rotavirus: This 70 nm, double shelled virus is now considered the most common cause of diarrhoea in young children. It has been detected in all countries where it has been looked for, though differences in prevalence exist from place to place. It exhibits a predilection for winter, even in the tropics where seasonal variations in temperature are not noticeable.

Rotavirus can frequently be demonstrated in the faeces of neonates, but in them the infection is generally asymptomatic, probably due to maternal Immunity. The disease peaks from the age of six months to two years. Most adults are immune, though subclinical infection may occur, particularly in contacts of infected children.

The mode of spread is believed to be faecal-oral. The Incubation period is 2–4 days. Typically, vomiting is a prominent early symptom, often preceding diarrhoea. The stools are watery, sometimes with flakes of mucus. Mild fever and respiratory symptoms may occur in a proportion of cases. Severity of diarrhoea is variable. Mortality is infrequent. The average duration of illness is 5–7 days.

Rotavirus infects the absorptive villous epithelial cells of the small intestine, causing replacement of the tall columnar epithelium with cuboidal cells, shortening of villi and lymphocytic infiltration of villous lamina propria. Diarrhoea may be related to a loss of absorptive capacity of the small intestine. Rotavirus diarrhoea is associated with a transient defect in the digestion and absorption of carbohydrates, but this does not limit the utility of rehydration with oral glucose electrolyte solution.

Rotavirus may cause sporadic diarrhoea as well as large epidemics. It has been found to cause very large annual 'winter' epidemics of childhood diarrhoea in north Kerala. Similar epidemics in Manipur have also been shown to be caused by the same agent.

Human rotavirus is a member of the family of viruses that cause diarrhoea in the young of many animals — calves, piglets, lambs, monkeys and mice. Although antigenic relationships exist among them, there is no evidence that human diarrhoea can be caused by any of the animal viruses. Human rotaviruses belong to different serotypes, at least four having been defined so far.

2. Norwalk virus: The Norwalk virus and related viruses (Hawaii, Montgomery county, Ditchling, "W") are 25–27 nm viruses which appear to cause diarrhoea, mainly in older children and adults. The reported outbreaks have occurred in schools, colleges, families and on board cruise ships. Foodborne outbreaks have been reported.

The incubation period is about 12-48 hours. Nausea, vomiting, abdominal discomfort, diarrhoea, mild fever and headache constitute the usual picture. The illness is mild and lasts usually for 1-2 days. The virus is shed in faeces and vomitus during the first three days of infection.

As the virus has not been propagated, information is scanty about its distribution. Antibodies to this group of viruses have been demonstrated in several countries. Prevalence of infection appears to be much less than with rotavirus. About 50 per cent of adults appear to be susceptible as judged by the absence of antibodies.

3. Adenoviruses: There have been several reports of diarrhoeal disease in children, where adenovirus could be demonstrated in large numbers in stools by electron microscopy, but could not be cultivated. Adenovirus types 40 and 41 have been identified in such cases.

4. Other viruses: Several reports have appeared about the association of diarrhoeal illness with the electron microscopic demonstration in faeces of many kinds of viruses, including astroviruses, caliciviruses and coronaviruses. More informa-

tion has to be obtained about the nature and significance of these viruses.

C. Protozoa

1. Entamoeba histolytica: Amoebic dysentery is endemic in many parts of the world. Human cyst passers constitute the source of infection. Food and drink are the usual vehicles, though person to person transmission may also occur. During the acute disease, stools contain only trophozoites and not the cystic form. This stage is not infectious.

The infection can become chronic and also lead to extraintestinal manifestations.

Recent evidence suggests that there are four types of *E. histolytica*, only one of which is pathogenic.

2. Giardia lamblia: Many types of diarrhoeal illness have been observed following infection with *Giardia lamblia*. These include explosive watery diarrhoea, loose foul smelling stools, and steatorrhoea. Abdominal cramps, anorexia and flatulence are common.

Asymptomatic cyst passing carriers form the source of infection. Infection may take place through food and drink, or by direct person to person spread. Giardiasis may account for some cases of travellers' diarrhoea. It was reported to be common in visitors to the Soviet Union.

3. Cryptosporidium, a coccidian parasite causing enteritis in calves and a variety of other animals and birds, has been found to cause diarrhoea in man, particularly in the immunodeficient and those in close contact with animals.

4. Balantidium coli: This, the largest intestinal protozoon of humans, is a rare cause of chronic recurrent diarrhoea, with dysenteric episodes in some.

D. Fungus

Candida albicans: There have been a number of reports recently of diarrhoea associated with a large number of *Candida albicans* in faeces. Some of these have been secondary to oral administration of antibiotics, but many cases have been apparently due to primary infection. The pathogenesis of the disease is not known.

FOOD POISONING

Unwholesome food may lead to illness or even death. The food may, in itself, be toxic or it may contain organic or inorganic poisons. Food also serves as a vehicle for several types of infections. Food poisoning may be chemical or microbial.

The term 'microbial food poisoning' has been interpreted in many ways. British workers have traditionally defined bacterial food poisoning as acute gastroenteritis due to the bacterial contamination of food and drink. But paradoxically, shigellosis was not included in this category, though it could often be foodborne, and botulism was considered along with food poisoning though diarrhoea is not present in the condition. American workers sometimes include many extraintestinal foodborne infections also under food poisoning, for example trichinellosis. It appears reasonable to restrict the definition of microbial food poisoning to diarrhoeal diseases caused by consumption of food contaminated with microorganisms or their products. This excludes foodborne conditions such as botulism and mycotoxicosis in which diarrhoea is not a feature. Food allergies and idiosyncrasies also do not qualify as food poisoning.

Traditionally, food poisoning has been classified into three types, depending on pathogenesis:

1. Infection type, where infective doses of pathogenic microorganisms are ingested with food. They set up infection in the gut and induce diarrhoea. Incubation period is generally 8–24 hours. The typical example is food poisoning by salmonellae.

2. Toxin type, where preformed bacterial toxin is ingested with food, as in staphylococcal food poisoning. The toxin induces vomiting and diarrhoea. Incubation period is short, 1–6 hours.

3. Intermediate type, where the bacteria ingested with food elaborate the toxin in the gut, which induces diarrhoea. The incubation period is 6–12 hours. The typical example is *Cl.perfringens* food poisoning.

With advances in understanding of the pathogenesis of diarrhoea, the distinctions between the above types are getting blurred. Enterotoxins are now known to be produced by several species of bacteria. As enterotoxin production and capacity for intestinal colonisation can be plasmid borne, these are not delimited by strict genus or species barriers.

Food poisoning is an epidemiological concept and not an aetiological one. Most of the microorganisms described above can cause food poisoning. There appears to be geographical differences in the relative prevalence of different microbial agents in food poisoning. In Britain, salmonellosis accounts for most outbreaks, while in the USA staphylococcal food poisoning has been the commonest type. In Japan, most cases are caused by *Vibrio parahaemolyticus*. Such differences may, in part, be due to differences in food habits. There is little aetiological information about food poisoning in the developing countries. In India, most recorded outbreaks have been caused by salmonellae.

Food poisoning always affects a number of people. Outbreaks may involve members of a family, persons attending a party or feast, inmates of a hostel, or unrelated persons taking food from a hotel. Sometimes persons in different cities or even different countries may be involved in an outbreak caused by some widely distributed product such as egg powder or tinned foods.

Diagnosis of food poisoning is primarily clinical. A detailed history including food articles consumed, persons affected and the sequence of development of symptoms would often give a clue regarding the source of infection and probable aetiological agents. Specimens to be collected for laboratory investigation include stools of patients and samples of suspected food. Vomitus is much less useful. Paired samples of blood may help demonstrate serological diagnosis of infection. In case of death, samples of intestinal contents, spleen, liver and heart blood may be collected.

For establishing the aetiological agent, it should be demonstrated in the stools of patients and in the food consumed. As some potential pathogens such as staphylococci and *Bacillus cereus* are normally present in stools and many items of food, a quantitative assessment is necessary. Very large numbers are significant.

Examination of stained smears of the specimen is useful to demonstrate heavy contamination with staphylococci, *B. cereus* or *Cl. perfringens*. Cultures are put up on appropriate media. Selective and enrichment media are essential. In some cases, the diagnosis may be confirmed by demonstrating rise in titre of antibodies in the patient. The primary source of infection may be difficult to identify. When food handlers are suspected to be carriers of staphylococci or salmonellae, cultures of their nasal swabs or stools would be necessary.

Prevention of food poisoning depends on strict attention to cleanliness at all levels of preparation, storage and serving of food.

MANAGEMENT OF DIARRHOEAL DISEASES

The management of acute diarrhoeal diseases has undergone a revolutionary change in recent years. This has followed the recognition that whatever be the cause of diarrhoea, its immediate danger is acute loss of water and electrolytes, and long term danger, nutritional deficiency. So the major objectives in management are very early replacement of water and electrolytes to prevent or treat dehydration, and the maintenance of adequate nutrition. Provided these are done, the body's defences will almost always control all cases of bacterial and viral diarrhoeas. Specific chemotherapy is essential only for protozoal diarrhoeas.

In order to simplify and standardise rehydration treatment in diarrhoea, the World Health Organisation has introduced the oral rehydration fluid, which has the following composition:

Sodium chloride 3.5 g
Sodium bicarbonate 2.5 g
Potassium chloride 1.5 g
Glucose 20 g, to be dissolved in one litre of potable water. The powders can be made up in packets and stored.

The scientific basis of this formulation is the observation that glucose enhances the absorption of electrolytes in the gut. The composition is so adjusted that it matches the electrolyte loss in watery diarrhoeas, enterotoxic or viral. Young babies with enteritis may not lose this much sodium, but when the kidneys are functioning normally, the body makes its own adjustments. However, in young babies it is advisable to alternate feeds of oral rehydration solution with half the quantity of plain water. In general, thirst is a good feedback for oral rehydration therapy and as much fluid should be given as is necessary to keep the patient free from thirst. In the large majority of patients, oral rehydration is all that is necessary. Intravenous fluids are called for only when patients present with severe dehydration. When the emergency is tided over, further rehydration can be continued orally. Vomiting is no contraindication to oral rehydration.

Alongside the correction of dehydration, it is important to maintain feeding. Breastfed babies should continue to receive breast milk. As temporary lactose intolerance is common in babies with diarrhoea, cow's milk should be given well diluted. If this worsens the diarrhoea, milk may be replaced by protein foods. Staple foods such as cooked cereals or legumes can be continued during the diarrhoea. Adults should resume normal diet as soon as appetite returns.

The so called 'routine' antibiotics have no place in the management of uncomplicated diarrhoeal illnesses. Tetracycline was formerly recommended for the treatment of cholera as it was found to reduce the need for intravenous fluids. But with the popularisation of early oral rehydration and the spread of R factors, this recommendation has been withdrawn. Antibiotics do not help in cases of uncomplicated salmonellosis or shigellosis; on the other hand, they tend to prolong the duration of faecal shedding of these bacteria. Moreover, multiple drug resistance is so common in *E. coli*, salmonellae, shigellae and other intestinal Gram negative bacilli that blind therapy with antibiotics is a waste. Antibiotics, are, of course, useless in viral diarrhoea. The only instances where antibiotics are indicated are when there is evidence of systemic infection with fever, toxicity or shock. The 'routine' use of antibiotics in diarrhoea has been mainly responsible for the dissemination of R plasmids conferring multiple drug resistance among intestinal Gram negative bacilli.

'Binding agents' and inhibitors of peristalsis have not been shown to be useful in the treatment of diarrhoeal diseases.

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67 Laboratory Control of Antimicrobial Therapy

Antibiotic sensitivity tests

Apart from rare exceptions like *Strep. pyogenes*, pathogenic bacteria exhibit very great strain variations in susceptibility to antibiotics and chemotherapeutic agents. This is particularly marked in the case of *Staph. aureus* and Gram negative bacilli. Therefore it is essential to determine the susceptibility of isolates of pathogenic bacteria to antibiotics that are likely to be used in treatment. Antibiotic sensitivity tests are of two types — diffusion tests and dilution tests.

Diffusion tests: Here the drug is allowed to diffuse through a solid medium so that a gradient is established, the concentration being highest near the site of application of the drug and decreasing with distance. The test bacterium is seeded on the medium and its sensitivity to the drug determined from the inhibition of its growth. Several methods have been used for the application of the drug. It may be added to ditches or holes cut in the medium or to hollow cylinders (Heatly cups) placed on it. But the method most commonly employed is to use filter paper discs impregnated with antibiotics.

The 'disc diffusion' method uses filter paper discs, 6.0 mm in diameter, charged with appropriate concentrations of the drugs. The discs are stored dry in the cold. They may be prepared in the laboratory or purchased commercially. A suitable dilution of a broth culture or a broth suspension of the test bacterium is flooded on the surface of a solid medium (Mueller-Hinton agar or nutrient agar). The plate is tilted to ensure uniform spreading and the excess broth pipetted off. Inoculation may also be performed by spreading with swabs. After drying the plate (37°C for 30 mins), antibiotic discs (four or five per 10 cm plate) are applied with sterile forceps. After overnight incubation, the degree of sensitivity is determined by measuring the zones of inhibition of growth around the discs. Growth will be inhibited around discs containing antibiotics to which the bacterium is susceptible, but not around those to which it is resistant.

The diameter of the zone of inhibition is influenced by a variety of factors, such as diffusibility of the drug, the disc concentration, the nature and composition of the medium, its thickness, presence of inhibitory or stimulatory substances, pH and time of incubation. It is therefore necessary to standardise all the variables. It is also necessary to check the potency of the discs periodically using as control a standard bacterium of known sensitivity, such as *Staph. aureus* Oxford strain (N.C.T.C. 6571).

There are several recommendations regarding the antibiotic concentrations to be used in discs. The Kirby-Bauer and the I.C.S. methods are in common use. Table 67.1 shows the disc concentrations and the critical zone sizes for antibiotics in common use.

A suitable method for routine use in diagnostic laboratories is the technique originally described by Stokes. This incorporates built-in controls against many variables and therefore provides dependable results. A standard sensitive strain of bacterium is inoculated in the middle third of the culture plate. The standard strains used are *Staph. aureus* ATCC 25923, *E. coli* ATCC 25922 or *Ps. aeruginosa* ATCC 27853, depending on the

68 Immunoprophylaxis and Immunotherapy

An important contribution of microbiology to medicine has been the development of immunisation methods for the prophylaxis and treatment of diverse diseases. Empirical immunoprophylaxis of smallpox in the form of variolation was practised over a thousand years ago. This was replaced by Jennerian vaccination which eventually led to the global eradication of the disease. Deliberate development of immunoprophylaxis started with Pasteur's discovery of vaccines for anthrax and rabies. The identification of causative agents of infectious diseases, one by one, and advances in techniques for their cultivation led to the development of vaccines against most of them.

The demonstration, towards the end of the last century, that antibodies specifically neutralised bacteria and their toxins led to the production of specific antisera for the prophylaxis and treatment of many infectious diseases. Immunoprophylaxis and therapy have also been extended to some noninfectious diseases such as malignancy. Immunopotentiation or enhancement of nonspecific immunity has also been practised. While attention has been centred largely on humoral immunity, local immunity and cell mediated immunity have been identified as important in some instances. Immunological restitution has also been possible for providing immunological support in cases of immunodeficiencies. A paradoxical application of immunisation is in the use of antilymphocyte serum for immunosuppression.

ACTIVE IMMUNISATION

Active immunoprophylaxis can be considered under two headings: 1) Routine immunisation of children which forms part of basic health care of communities, and 2) immunisation of individuals or selected groups exposed to risk of particular infections.

Routine immunisation schedules have been developed for different countries, based on the prevalence of infectious diseases, their public health importance, availability of suitable vaccines, their adverse reactions, cost benefit factors and logistics. The 'Expanded Programme on Immunisation' recommended by the World Health Organisation for the developing countries is directed against tuberculosis, diphtheria, pertussis, tetanus, poliomyelitis and measles. In the developing countries, 0.5 per cent of all newborns can be expected to become crippled by poliomyelitis; one per cent to die from neonatal tetanus; two per cent from pertussis; and three per cent from measles. Some five million children die from these diseases each year, 10 children every minute.

A recommended schedule of immunisation for India and other developing countries is shown in Table 68.1.

BCG vaccine

Though BCG vaccine has been in use for about 60 years, its efficiency is still questioned. Several field trials have been carried out, with protection rates ranging from nil to 80 per cent. No protection against pulmonary tuberculosis in adults was observed in a large field trial near Madras recently. It is however considered that BCG may be useful in infants and children, particularly against the more serious types of infection.

TABLE 68 1
Schedule of immunisation

Visit	Age in months	Vaccine
1	0	BCG
2	2	DPT (1) Oral poliovaccine (1)
3	4	DPT (2) Oral poliovaccine (2)
4	6	DPT (3) Oral poliovaccine (3)
5	9	Measles Oral poliovaccine (4)
6	18	DPT (4) Oral poliovaccine (5)
7	School entry (5 years)	Tetanus toxoid

(However the Indian Academy of Paediatrics recommends the following immunisation schedule: BCG 0–12 months; DPT/OPV 6 weeks to 9 months. Three doses only at an interval of four weeks, Measles: 9–12 months;

Repeat doses: DPT/OPV: 18 months to 24 months; DT. 5–6 years, one dose. Typhoid 5–6 years, two doses at four weekly interval; TT/Typhoid 10 years and 15 years one dose each. Pregnant women. TT two doses at interval of six weeks.)

meningitis and miliary tuberculosis. It is therefore recommended that the vaccine be administered to infants, soon after birth, before mother and baby are discharged from hospital. Only cell mediated immunity is relevant in tuberculosis and therefore the newborn baby is competent to respond to BCG vaccine. Booster doses of BCG are not usually administered because in the developing countries natural infections with tubercle bacilli maintain the immunity. In countries where tuberculosis is rare, immunity following a single dose of BCG wanes during the next several years and a booster dose may be indicated at school entry.

Complications are few and consist of local ulceration, enlargement and suppuration of draining lymph nodes, and keloid formation. Progressive infection may occur in immunodeficient individuals.

It has been reported that BCG may afford some protection against leprosy and leukaemia.

The vaccine is manufactured at the BCG Laboratory, Guindy, Madras.

DPT vaccine (Triple antigen)

Diphtheria, pertussis and tetanus are very important childhood infections which can be prevented by immunisation. Diphtheria used to be a rare disease in the developing world as immunity was obtained early from diphtheritic skin infections However, with urbanisation, incidence of the disease has increased enormously. The mortality from diphtheria in the developing countries is ten to twenty times higher than in the developed countries. Pertussis affects approximately 80 per cent of all children and has a mortality rate of 1–3 per cent. It is a debilitating illness, often paving the way for secondary infections. Tetanus is very common in the developing world, affecting particularly children in rural areas. It is one of the commonest causes of childhood mortality in the tropics.

 adjuvant for the toxoi

DPT vaccine contains the toxoids of diphtheria and tetanus and a killed suspension of pertussis bacilli. The component antigens can be given separately also, but this has no advantage. On the other hand, giving them together not only minimises the number of injections, but also improves the immune response because the pertussis vaccine acts as an adjuvant for the toxoids. The primary course of DPT immunisation consists of three subcutaneous injections, with an interval of 4–8 weeks between the first and second, and 8–12 weeks between the second and third doses. This schedule provides a better antibody response than the traditional schedule of three injections at monthly intervals. In India and in other developing countries, it is advisable to start DPT immunisation at the age of two months. Diphtheria is rare in children under six months as they are protected by maternal antibodies till then. Tetanus in the newborn can be prevented by immunising pregnant women with tetanus toxoid. It would therefore have been enough to start immunisation at six months of age, but for the fact that there is little maternal immunity against pertussis, which can be a very severe illness in young babies. This is the reason for recommending commencement of DPT immunisation at two months. Earlier immunisation may not be effective due to immaturity of the immune system. A booster dose is recommended at 18 months of age. Diphtheria and pertussis are uncommon after five years, but tetanus can occur at any age. Therefore a booster of tetanus toxoid is advisable at school entry. This should provide life long protection.

Adverse reactions to DPT vaccine are rare and consist of transient local inflammation, fever and occasional convulsions. Because of the possibility of provocative poliomyelitis, it is advisable not to carry out routine immunisation if poliomyelitis is active in the area.

Because diphtheria is still prevalent in many parts of the world, a booster injection of diphtheria toxoid for adults is indicated. This is usually given in combination with tetanus toxoid purified for adults.

Poliomyelitis

Two effective vaccines are available against poliomyelitis, the live oral vaccine (OPV) and the inactivated parenteral vaccine (IPV). Oral polio vaccine is ideally suited for mass immunisation. Three doses of trivalent OPV have regularly induced very satisfactory levels of antibody against all three types of polioviruses in the advanced countries in temperate climes. This dosage schedule was widely used in the tropics also, but seroconversion rates in India and many other tropical countries have been poor. There have been many cases of paralytic poliomyelitis recorded in children who have received three doses of OPV. Many reasons have been proposed for this poor response, including poor potency of the vaccine due to improper storage, interference by other enteroviruses in the gut, neutralisation by antibodies in breast milk and presence of inhibitors in gastrointestinal secretions. Suggested remedies have included the use of high potency vaccine, or horse serum against human gammaglobulin to neutralise inhibitors, avoidance of breast feeding for some hours before and after administration of vaccine, increasing the number of doses to five, and simultaneous vaccination of all infants and children in one locality. It has also been proposed by some that OPV may not be suitable for the developing countries and that IPV may be used instead. Though IPV is costlier and the logistics of administration difficult, it has been suggested that it may be incorporated with DPT and given as a quadruple vaccine. IPV is not available in India now. For the present, the best course appears to be to give five doses of OPV as suggested in the schedule.

IPV is recommended for immunodeficient children, children with immunodeficient members in the household and for those initially immunised after the age of 18.

Measles

Measles is the commonest exanthematous disease of childhood. While it is generally a minor

illness in temperate regions, it can be a very serious illness in the tropics. Live attenuated measles vaccines have been found to be very effective in prophylaxis. The earlier vaccines were associated with unacceptable febrile reactions (vaccination measles) but more attenuated vaccines are now available which do not cause any serious side effects. The vaccine is given as a single dose of *subcutaneous injection* in children 9–12 months old. Younger infants usually possess some maternal antibodies and vaccination in them may prove ineffective. Booster injections are not necessary. There have been reports of subacute sclerosing panencephalitis in children immunised with measles vaccine, but the incidence is far less than that following natural measles. Therefore, vaccination may actually reduce the chance of SSPE developing, by preventing natural measles.

Measles vaccine is not manufactured now in India and so its availability is limited.

Rubella

The indication for rubella vaccine is not so much to protect the vaccinees as to prevent the danger of infection in pregnant women, leading to fetal malformations. Therefore vaccination is relevant only in women. Different strategies of vaccination have been used in different countries, such as immunising all children or only prepubertal girls. Surveys done in different parts of India have shown that 80–85 per cent of women in the childbearing age are immune to rubella. But even the residual nonimmunes constitute such enormous numbers with a potential danger of bearing malformed babies, that vaccination is indicated for them.

Arthralgia is a frequent complication following vaccination.

T.A.B. vaccine

The monovalent typhoid vaccine was introduced by Wright in 1897. It was believed to have brought down the incidence of typhoid fever in troops compaigning in South Africa. During the First World War, when British troops had to serve in Europe, Africa and Asia, where typhoid and paratyphoid fevers were common, the typhoid vaccine was supplemented with killed paratyphoid A and B bacilli, in the hope that the polyvalent T.A.B. vaccine would protect against typhoid and paratyphoid fevers. While field trials with monovalent typhoid vaccines have shown fair degrees of protection, no such trials have been carried out with the polyvalent T.A.B. vaccine. It is generally stated that immunity lasts for only six months after the vaccine, but there is much evidence that in endemic areas, immunity may last for some years. Of the several types of typhoid vaccines, the acetone killed and dried vaccine was found to be the most effective. The heat killed phenolised vaccine used in India is also effective.

Because of the rarity of paratyphoid B in India, this component of T.A.B. vaccine has been deleted. The vaccine manufactured at present *contains* only typhoid and paratyphoid A *bacilli*. It is at present not used routinely in the general population, but only in special groups such as the armed forces, nurses and other hospital workers, in whom it is given annually. Mass immunisation is sometimes practised when a large outbreak of enteric fever occurs in an area. It is sometimes given to household contacts of patients with enteric fever. The safety of this last procedure is questionable. The concept of the 'negative phase', a transient period of decreased antibody level immediately following administration of the vaccine, once considered important, has tended to be forgotten later on. There is some recent evidence that the vaccine induces a transient depression of cell mediated immunity to the typhoid bacillus. As cell mediated immunity is being increasingly recognised as the more relevant type of immunity in enteric fever, this finding may be important.

Though not recommended for routine mass immunisation, it may be desirable to give two *doses of typhoid vaccine subcutaneously* at an interval of one month, to children at about the age of two years. A booster at school entry may

be helpful. The immunity may be expected to be augmented by repeated natural subclinical infections

A more effective typhoid vaccine is called for. Oral immunisation with live attenuated bacillus would seem to be more appropriate.

The live oral typhoid vaccine prepared from the Ty21a mutant strain proved very effective in a clinical trial in Egypt. A subsequent trial in South America was not so successful. Its administration is cumbersome requiring prior neutralisation of gastric acidity.

Cholera

The killed parenteral cholera vaccine is used widely in India and other developing countries. It had been shown to provide limited protection for upto six months only. Recent trials in Calcutta and in Indonesia with an aluminium phosphate adjuvant vaccine have yielded much better protection for about two years, particularly in young children in whom the plain vaccine was not useful. But the adjuvant vaccine has not been manufactured for routine use as yet.

At present, the cholera vaccine is often misused. Whenever any outbreak of gastroenteritis is reported in an area, cholera vaccination is begun. Many of these outbreaks may not be cholera at all. Even if the outbreak is due to cholera, the vaccine given during the epidemic does little good. On the other hand, such mass immunisations have sometimes led to serious complications due to lapse in asepsis. The vaccine may be of use in endemic areas, before the annual epidemic cholera season begins.

Influenza Monovalent
Polyvalent

Inactivated influenza virus vaccines, monovalent or polyvalent, are used in Britain annually before the onset of winter, in special groups such as the elderly with chronic bronchitis. This helps reduce morbidity and mortality among them. But in the tropics, where annual winter epidemics of influenza are not a feature, routine immunisation is not called for. Influenza immunisation becomes necessary only when a pandemic spread is anticipated.

Mumps

The live vaccine is available in the advanced countries. This may be given either alone, or in combination with measles and rubella vaccines. It is however not needed in the developing countries, where mumps is a minor illness.

Other vaccines

Vaccines have been produced with most of the pathogenic microbes affecting man and animals. But, apart from the vaccines discussed above, few are used widely. Some vaccines have become redundant when effective chemotherapy became available, as for example the pneumococcal vaccine. A polyvalent pneumococcal polysaccharide vaccine containing the common serotypes is used in the USA in selected vulnerable persons. Meningococcal vaccines containing the protective polysaccharide antigens have been employed against epidemic meningococcal meningitis. Live avirulent shigella strains have been proposed for oral immunisation, but they have not been cleared for general use.

Plague vaccine is now seldom used. In India the only vaccine available is the Sokhey's modification of the Haffkine vaccine, manufactured at the Haffkine Institute, Bombay.

Killed *Pseudomonas aeruginosa* vaccines have been used in severely burnt patients and some reports have claimed good results.

A live chickenpox vaccine developed in Japan is recommended in leukaemic and other high risk children

The 17 D yellow fever vaccine is manufactured at the Central Research Institute, Kasauli. As yellow fever does not occur in India, the vaccine is used only for persons travelling to endemic areas.

An effective vaccine has been developed in Japan for Japanese encephalitis. As the disease

has now become a serious problem in many parts of India, vaccination may become necessary. The vaccine is being manufactured at the Central Research Institute, Kasauli.

An inactivated vaccine for Kyasanur Forest disease has been developed in India. Though pilot studies have yielded encouraging results, the vaccine has not been manufactured in bulk, nor mass immunisation attempted, because of the very limited area and population involved by the infection.

Rabies vaccines still commonly used for human immunisation in India are of the neural type, either Semple or BPL vaccines. As these carry the risk of neuroparalytic complications, vaccination is practised only postinfection. Vaccination would be more effective if it is done before infection has taken place. The cell culture vaccine is safe and effective for pre- and postexposure immunisation. But its high cost limits its utility.

An inactivated hepatitis B virus vaccine derived from human carrier plasma has been licensed for use in selected groups at high risk. But it is costly and availability limited. Recombinant heptatitis B vaccines have been developed.

An experimental attenuated virus vaccine is currently undergoing clinical trials in humans.

Autovaccines

For some chronic or recurrent infections not responding to antibiotics, autovaccines have been found to be useful. The infecting strain of bacterium is grown and graded doses of a suspension of the bacterium killed by heat and phenol are administered. Staphylococci and streptococci are the bacteria commonly used for autovaccine preparation.

Stock vaccines, consisting of killed preparations of standard strains of bacteria, were formerly used for some chronic conditions. They are seldom used now.

PASSIVE IMMUNISATION

Antisera against microorganisms and their toxins were widely used before advances in antibiotic and chemotherapy rendered antibacterial sera largely redundant. The sheet anchor in the treatment of pneumoccoccal pneumonia was the anticapsular antiserum till it was supplanted by sulphonamides and penicillin. Antitoxic sera and, to a less extent, antiviral sera are still in use for prophylaxis and therapy.

The antitoxic sera in common use are antidiphtheritic, antitetanus and antigasgangrene sera. Active immunisation is the best method for preventing diphtheria and tetanus and it can render antitoxins unnecessary for prophylaxis. Antidiphtheritic serum is rarely used for prophylaxis now, but antitetanus serum continues to be used extensively. This is unfortunate. Apart from serious complications such as anaphylaxis, the efficacy of ATS is poor when used repeatedly in the same subject. Casualty departments of many hospitals routinely use ATS for any injury. This practice needs to be revised and the use of ATS in prophylaxis restricted to tetanus prone wounds in the nonimmune. Human antitetanus globulin is safer and more effective than ATS of animal origin.

Antitoxic sera are invaluable in the treatment of the established disease. However, there is controversy regarding the utility of ATS in the treatment of tetanus.

As active immunisation against gas gangrene is not available, antigasgangrene serum still has a place in cases of crush injury.

Antisera against *Pseudomonas aeruginosa* have been tried in severely burnt patients.

Antisera have been used for the prophylaxis and treatment of many viral diseases. Antirabic serum is a very valuable adjunct in the immunoprophylaxis of rabies in patients with severe exposure to the virus. Convalescent sera or specific immune globulin had been employed for protection of contacts against smallpox, chickenpox, measles and hepatitis. Human gammaglobulin preparations contain antibodies against all common viral infections and have been used as broad spectrum antiviral immunoprophylactics. Type B hepatitis is a complication in the use of human sera and serum preparations.

Vaccination requirements for international travel

Different countries have various vaccination requirements for international travellers. Most countries insist on yellow fever vaccination and a few still require cholera vaccination. Smallpox vaccination is no longer needed. The WHO publishes periodically the vaccination requirements in force in different countries.

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69 Hospital Infection

Hospitals have always acted as a source of infection to patients admitted to them. Suppuration and gangrene were common postoperative consequences in hospitals in the last century. Even before the microbial aetiology of infections was established, Semmelweiss was able to control puerperal sepsis by simple hand washing, and Lister overcame surgical infections with phenol sprays. The concept of asepsis and its application in hospital practice reduced their incidence, but hospital infections still cause considerable morbidity and mortality. The incidence of hospital infection has been reported to be 2–12 per cent in the advanced countries; it is much higher in the crowded hospitals in the developing countries. Even when hospitalisation does not lead to obvious infection, it causes a change in the patient's microbial flora, the normal flora being gradually replaced by the drug resistant microorganisms typical of the hospital environment.

The terms *hospital infection, hospital-acquired infection* or *nosocomial infection* (from *nosocomeion,* meaning hospital) are applied to infections developing in hospitalised patients, which were not present or in incubation at the time of their admission. Such infections may become evident during their stay in hospital or, sometimes, only after their discharge. Hospital infections are typically exogenous, the source being any part of the hospital ecosystem, including people, objects, food, water and air in the hospital. Such infections may be iatrogenic in that they may be induced by some diagnostic or therapeutic intervention in the hospital. They may be opportunistic in that microorganisms of low virulence may cause disease in hospitalised patients whose immune mechanisms are impaired. But it must be understood that nosocomial infections are not synonymous with iatrogenic or opportunistic infections, as the latter may occur outside hospitals also.

Several factors contribute to the occurrence and severity of hospital infections: 1) Many patients in hospitals have impaired defence mechanisms due to their disease or the therapy administered. They are, therefore, highly susceptible to infection. 2) The hospital environment is heavily laden with a wide variety of pathogens. Patients shed them from their bodies; hospital personnel spread them through their hands and clothes. Bedding, linen and utensils act as fomites. Equipment may be contaminated. Pathogens are present in the hospital dust and air, and sometimes even in antiseptic lotions and ointments. Contamination of hospital food or water may cause outbreaks of infections. 3) Major invasive procedures, diagnostic or therapeutic, are carried out only in hospitals. The slightest lapse in asepsis during these procedures can lead to infection. 4) Hospital infections are generally more serious and refractory to treatment as the infecting agents are resistant to most antibiotics in common use. 5) Hospital infections are in a sense diseases of medical progress. Advances in treatment of cancer, organ transplantation, implanted prostheses and other sophisticated medical technologies enhance the risk of infection to patients.

Microbiology of hospital infections

Almost any pathogen can, on occasion, cause

hospital infection, but those that are able to survive in the hospital environment for long periods and develop resistance to antibiotics and disinfectants are particularly important in this respect. *Strep. pyogenes* was, perhaps, the most important cause of hospital infection formerly, but is hardly ever encountered now as it is highly susceptible to antibiotics. *Staph. aureus* strains, resistant to multiple antibiotics and belonging to phage type 80/81, spread globally in the 1950's and 1960's, colonising hospitals and causing nosocomial infection with such frequency that they came to be called 'hospital staphylococci'. The original phage types have since been replaced by others belonging to group IIf, but staphylococci continue to be very common agents in hospital infection. *Staph. epidermidis* and Group D streptococci also are sometimes responsible for hospital infections.

In recent decades, the enteric Gram negative bacilli — *E. coli*, *Klebsiella*, *Enterobacter*, *Proteus* and *Serratia* — have become the most important group of hospital pathogens, particularly following the dissemination among them of R factors conferring multiple drug resistance. During the late 1970's multidrug resistant salmonellae, particularly *S. typhimurium*, became a prominent hospital pathogen.

Ps. aeruginosa and other *Pseudomonas* species have always been important causes of hospital infection because of their intrinsic resistance to most antibiotics and ability to survive and even multiply at low temperatures and in disinfectant solutions. They may also carry drug resistant plasmids.

Tetanus spores can survive in dust for a very long time and may sometimes contaminate cotton, suture materials, plaster of paris and other items used in hospitals. Hospital tetanus is usually a result of faulty sterilisation techniques or other lapses in asepsis.

Hepatitis type B is probably the most important virus causing hospital infections, being transmitted by blood transfusion or other procedures involving transfer of blood, plasma or body fluids. Due to its long incubation period, the hepatitis may be evident only long after discharge from hospital. Screening of blood donors has reduced the incidence of post-transfusion type B hepatitis, but Non-A: Non-B hepatitis continues to occur.

Viral diarrhoea and chickenpox are other viral infections that spread in hospitals. Cytomegalovirus, herpesvirus, influenza, enteroviruses and arenaviruses may also cause hospital infection.

The range of hospital pathogens also includes yeasts *(Candida albicans*, principally), moulds, (Aspergillus, Mucor) and protozoa *(Entamoeba histolytica, Pneumocystis carinii, Toxoplasma gondii)*.

Common types of hospital infections

1. Wound infection: This may range in severity from delayed wound healing or stitch abscess caused by *Staph. epidermidis* or other resident skin flora, to severe spreading infections due to exogenous pathogens. Several factors influence the occurrence of postoperative wound infections, such as the site and duration of surgery, health of the patient and skill of the operator. Most wound infections manifest within a week of surgery. *Strep. pyogenes* and clostridial infections appear within a day or two, while staphylococcal infections typically take four or five days and Gram negative bacillary infections six or seven days to appear. Routine preoperative antibiotics do not prevent wound infections, though they may sometimes be delayed. In special cases where antibiotic cover is indicated, it should be given parenterally immediately before, during and immediately following surgery.

Nonsurgical sites of wound infections include injection, 'cut-downs', umbilical stumps, ulcers and burns. *Ps. aeruginosa* is the most important cause of infection in burns.

Tetanus as a result of hospital infections is now rare, but should be kept in mind and toxoid administered to nonimmune patients before elective surgery. Many cases of neonatal tetanus have occurred due to the use of contaminated umbilical cord ties.

2. Urinary tract infections: Even with adequate precautions, catheterisation in hospitals leads to urinary infections in about two per cent; with indwelling catheters, the rate goes upto 50 per cent or more. *E. coli, Proteus, Ps aeurginosa* and other Gram negative bacilli are the causative agents. Mixed infection is common. Infection can be prevented by strict asepsis during catheterisation. Indwelling catheters are to be used only when unavoidable, and then only with proper closed drainage.

3. Respiratory infections: Aspiration in unconscious patients and pulmonary ventilation or instrumentation may lead to nosocomial pneumonia, particularly in those with preexisting cardiopulmonary disease. Multidrug resistant *Staph. aureus* and Gram negative bacilli are the common pathogens. Antibiotic treatment is unsatisfactory. Postural drainage is useful in the prevention and management of such cases.

4. Bacteraemia and septicaemia: These may be consequences of infections at any site, but are commonly caused by infected intravenous cannulae. The longer the cannulae are kept *in situ*, the greater the risk of infection. 'Cut-downs' on the leg veins in infants or children with diarrhoea generally get left in place for long periods, the site being bathed in diarrhoeal stools. Phlebitis sets in with consequent bacteraemia. Many a child admitted with diarrhoea thus dies of septicaemia. Gram negative bacilli are the common pathogens. 'Cut-downs' are safer on the arms than on legs. Intravenous rehydration in diarrhoea should be restricted to emergencies and should be replaced by oral fluids as early as possible. Infection can be prevented by proper skin toilet before 'cut-down' and the use of stainless steel needles instead of plastic cannulae.

Staph. epidermidis bacteraemia is seen commonly in patients with artificial heart valves. Bacteraemia in those with valvular defects may lead to endocarditis.

Diagnosis and control of hospital infection

Hospital infection may occur sporadically or as outbreaks. Aetiological diagnosis is by the routine bacteriological methods of smear, culture, identification and sensitivity testing. When an outbreak occurs, the source should be identified and eliminated. This requires the sampling of possible sources of infection such as hospital personnel, inanimate objects, water, air or food. Typing of isolate — phage, bacteriocin, antibiogram or biotyping — from cases and sites may indicate a causal connection. Obvious examples of sources of hospital outbreaks are nasal carriage of staphylococci by surgeons or Pseudomonas growing in hand lotion. Carriers should be suitably treated.

Sterilisation techniques have to be tested. The cause of infection may be a defective autoclave or improper technique such as boiling infusion sets in ward sterilisers. A careful analysis of the pattern of infection may often reveal the source, but sometimes it eludes the most diligent search.

It must be emphasised that control of hospital infection should be not merely a spasmodic exercise to be employed when an outbreak occurs, but rather a permanent ongoing activity in any large hospital. Every major hospital should have 'infection control teams' consisting of microbiologists, medical and nursing staff and hospital administrators. Besides investigating and controlling outbreaks, their functions include formulating appropriate guide lines for admission, nursing and treatment of infectious patients, surveillance on sterilisation and disinfectant practices, determining antibiotic policies and immunisation schedules, and educating patients and hospital personnel on infection control. Such measures help in reducing the incidence of hospital infections, even if they do not eliminate them altogether.

Unfortunately, in many hospitals, infection control is attempted by resorting to more and more of antibiotics. This is not only futile, but may even be positively harmful by encouraging selective colonisation by multiresistant pathogens. In the final

analysis, prevention of hospital infection rests on a proper understanding of aseptic practices and meticulous attention to hygienic principles. Sir William Osler's aphorism that 'Soap, water and commonsense are the best disinfectants' applies even today in the context of hospital infection.

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SUBJECT INDEX